INTERLEUKIN-3

Identification, characterization and molecular evolution

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INTERLEUKIN-3

Identification, characterization and molecular evolution

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Identificatie, karakterisatie en moleculaire evolutie

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aan Remmy,
Maaike, Eva, Kay en Tim

ter nagedachtenis aan mijn moeder
voor mijn vader

LIST OF ABBREVIATIONS

A	- adenine
aa	- amino acid
AET	- 2-aminoethylisothiouronium
AML	- acute myeloid leukemia
bp	- base pair
BM	- bone marrow
BFU-E	- burst forming unit-erythroid
BSA	- bovine serum albumin
C	- cytosine
cDNA	- complementary DNA
CFU	- colony forming unit
CFU-Baso	- colony forming unit-basophils
CFU-C	- colony forming unit-culture
CFU-E	- colony forming unit-erythroid
CFU-Eo	- colony forming unit-eosinophils
CFU-GM	- colony forming unit-granulocytes/macrophages
CFU-GEMM	- colony forming unit-granulocytes/erythrocytes/ macrophages/megakaryocytes
CFU-Meg	- colony forming unit-megakaryocytes
CFU-S	- colony forming unit-spleen
CM	- conditioned medium
ConA	- concanavalin A
CPM	- counts per minute
CSF	- colony stimulating factor
G-CSF	- granulocyte-colony stimulating factor
GM-CSF	- granulocyte/macrophage stimulating factor
M-CSF	- macrophage-colony stimulating factor
Multi-CSF	- multilineage-stimulating factor
DEAE	- diethylaminoethyl
DNA	- deoxyribonucleic acid
DMSO	- dimethylsulfoxide
DTT	- dithiothreitol
EDTA	- ethylene diamine tetra-acetate
Epo	- erythropoietin
FCS	- fetal calf serum
G	- guanine
hIL-3	- human interleukin-3
HGF(s)	- hemopoietic growth factor(s)
HLCM	- human leukocyte conditioned medium
HSC	- hemopoietic stem cell
IPTG	- isopropyl-β-d-thiogalactoside

JAK	- janus kinase or just another kinase
kb	- kilo base pair
kDa	- kilo dalton
IL(s)	- interleukin(s)
IFN(s)	- interferon(s)
LIF	- leukemia inhibitory factor
MCA or MAb	- monoclonal antibody
MGF	- mast cell growth factor (=SCF=*c*-kit ligand=steel factor)
mRNA	- messenger RNA
MSCM	- mouse spleen conditioned medium
nt	- nucleotide
OTU	- operational taxonomic unit
PAGE	- polyacrylamide gel electrophoresis
PBS	- phosphate buffered saline
PBL	- peripheral blood lymphocyte
PEG	- polyethylene glycol
PHA	- phytohaemagglutinin
PHSC	- pluripotent hemopoietic stem cell
PMSF	- phenylmethylsulfonylfluoride
PP_i	- sodium pyro-phosphate
RhIL-3	- rhesus monkey interleukin-3
SAF	- stem cell activating factor
SCF	- stem cell factor (=MGF=*c*-kit ligand=steel factor)
SD	- standard deviation
SDS	- sodium dodecyl sulphate
SE	- standard error
SSC	- standard sodium chloride / sodium citrate solution
SSPE	- standard sodium chloride / sodium phosphate / EDTA solution
STAT	- signal transduction activated transcription
SV40	- simian virus 40
T	- thymine
TGF	- tumor growth factor
TNF	- tumor necrosis factor
TPA	- 12-0-tertdecanoylphorbol-13-acetate
WSXWS	- tryptophan-serine-x-tryptophan-serine motif
X-gal	- 5-bromo-4-chloroindolyl-β-D-galatoside

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Hemopoiesis

Blood contains large numbers of various cell types. The mature blood cell types exert highly specialized functions such as oxygen and carbon dioxide transport, blood clotting and defense against infections by antibody production, cell mediated immunity and phagocytosis. Most of these mature blood cell types have a limited life span and therefore need to be produced continuously. This process of blood cell formation, termed hemopoiesis, is impressive since daily approximately 10^{11} new blood cells are generated in man (Wagemaker, 1985). In steady state situations, the continuous replacement of terminally differentiated cells is tuned with great precision but the hemopoietic system can respond dramatically to environmental stress, such as bleeding or infection (Metcalf, 1985). The primary site of hemopoiesis is the bone marrow which permits the formation of all blood cell types i.e., erythrocytes, platelets, monocytes, neutrophils, basophils, eosinophils and lymphocytes. The continuous replenishment of functionally mature hemopoietic cells *in vivo* is strictly dependent on the presence of a small but persistent pool of bone marrow pluripotent hemopoietic stem cells (see section 1.2).

The mechanism(s) controlling hemopoiesis appear to involve regulation mediated by a group of interacting specific glycoproteins designated hemopoietic growth factors. Furthermore, it has been implied that microenvironmental stromal cells support hemopoiesis as well (Dexter, 1982; Hunt et al., 1987). Several mechanisms through which stromal cells affect hemopoiesis have been postulated, i.e., a direct cell contact regulated mechanism (Zipori, 1981; Spooncer et al., 1986), secretion of CSFs (Zipori et al., 1984; Rennick et al., 1987a); expression of antagonists of differentiation-inducing factor(s) and/or self-renewal mediators (Zipori, 1981; Zipori and Lee, 1988).

1.2 Pluripotent hemopoietic stem cells and their progeny

All blood cells originate from a small population of pluripotent hemopoietic stem cells (PHSCs) that is formed during a short period in early embryonic life and thereafter maintains hemopoiesis throughout life by an extensive capacity for regeneration. The ability for self-renewal does not decline during the natural life span (Schofield, 1978). It has been recognized that a small number of pluripotent hemopoietic cells, present in bone marrow transplants is responsible for reconstituting and restoring all hematological functions in lethally irradiated recipients (Ford et al., 1956; Vos et al., 1956; Nowell et al., 1956). Convincing evidence for the existence of a PHSC, defined by the ability of self-replication, extensive proliferation and the ability to generate committed progenitor cells to give descendants in all hemopoietic differentiation lineages, was shown by transplantation studies using lineage markers such as radiation induced chromosomal translocations (Wu et al., 1967; Abramson et al., 1977).

Retroviral marking studies further demonstrated that individual PHSCs present at the time of engraftment are both necessary and sufficient for permanent hemopoiesis (Williams et al., 1984; Keller et al., 1985; Lemischka et al., 1986). Precursor cells of all types and stages of differentiation lineages have been identified along the pathway of multilineage differentiation from the PHSCs towards the functional peripheral blood cells.

Many investigators divide the hemopoietic system into three main compartments of increasing maturity and an associated loss of proliferative potential (Till and McCulloch, 1980: Ogawa et al., 1983). The first, the most primitive compartment, consists of pluripotent stem cells that have the extensive capacity of self-renewal as well as the capacity to generate primitive progenitors that are programmed to differentiate (commitment). The second compartment consists of committed progenitor cells that have the capacity to proliferate and differentiate in only one or two lineages. Cells in this compartment have lost the capacity of indefinite self-renewal and are responsible for maintaining the level of mature blood cells. The third compartment consists of mature cells with highly specialized and vital functions. Most of the cells in this compartment have lost there ability to proliferate, have a limited life span, and need to be continuously replenished. A schematic representation of hemopoietic stem cell differentiation is shown in Fig. 1.1

In 1961 Till and McCulloch described an assay for progenitor cells capable of forming hemopoietic colonies in the spleens of irradiated syngeneic mice. The number of spleen colonies was linearly related to the number of transplanted cells (Till and McCulloch, 1961). These colonies are derived from single cells (Becker et al., 1963; Wu et al., 1967) and contain red cells, granulocytes, megakaryocytes, and new colony forming cells (Siminovitch et al., 1963). Such an apparently immature cell that gives rise to a spleen colony was operationally termed colony forming unit/spleen (CFU-S). The spleen colony method provided a quantitative assay for murine multipotent hemopoietic stem cells, provisionally referred to as the CFU-S assay. Transplantation of a single spleen colony into a lethally irradiated recipient demonstrated reconstitution capacity by the development of a complete hemopoietic system (Trentin and Fahlberg, 1963; Till and McCulloch, 1980). In this regard the CFU-S resembles the murine PHSC and appeared to be of considerable value for experimental hematology. However, the pluripotency of CFU-S is still not clear because experimental data suggest that CFU-S comprises subpopulations that differ in respect to the development time of the examined colonies i.e., day-7-8 CFU-S and day-12-14 CFU-S. The different populations appeared to be associated with differences in self-renewal capacity (Siminovitch et al., 1963; Magli et al., 1982). Furthermore, the heterogeneous population of CFU-S consists of cells with different physical properties (Metcalf et al., 1971; Baines and Visser, 1983) and biological properties (Harris et al., 1984; Johnson and Nicola, 1984; Mulder et al., 1985; Mulder, 1986). Based on these

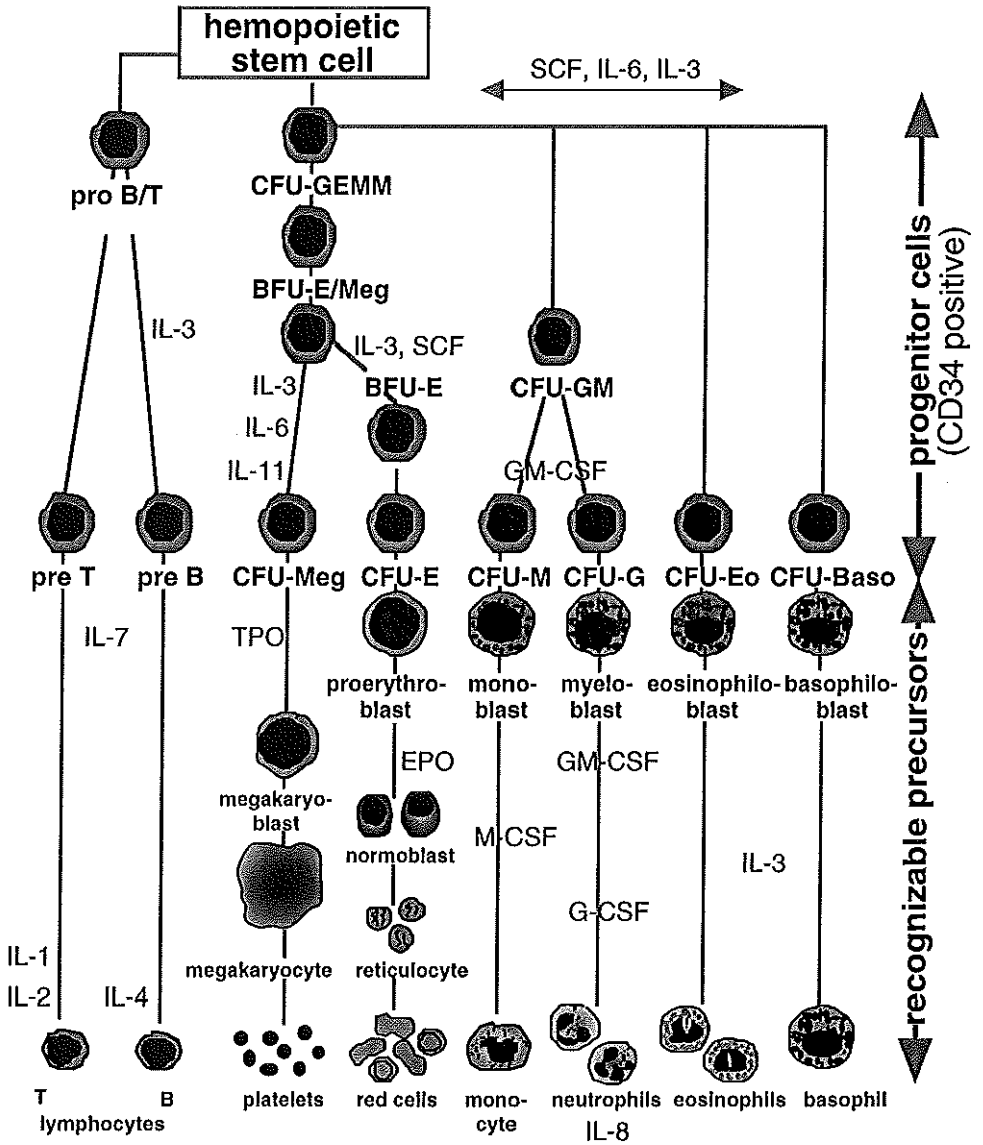

Fig. 1 Schematic representation of blood cell formation and hemopoietic stem cell differentiation with main sites of action of hemopoietic growth factors. See also Table 1.1

differences it was suggested that day-8 CFU-S is the progeny of day-12 CFU-S and that at least a proportion of the CFU-S (day-12-14 CFU-S) is closely related or even identical with the murine PHSC.

The discovery of conditions for clonal assay of murine hemopoietic precursor cells *in vitro* marked a major advance in the study of blood cell growth and differentiation (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). This system for culturing murine granulocyte/monocyte colonies in agar was soon modified for growth of human blood cell progenitors (Pike and Robinson, 1970). Since the CFU-S assay was obviously not applicable for hemopoietic progenitors of man or other outbred species, the development of semisolid clonal cell culture methods was the key to culture and quantitate committed hemopoietic human progenitor cells. Progenitors measured by these clonal assays represent a continuum of stages in each hemopoietic differentiation lineage and various intermediate progenitors have been described (reviewed by Ogawa et al., 1983 and Metcalf, 1986). Similar to the provisional term CFU-S, used to designate the cells that give rise to spleen colonies, such a progenitor cell has been termed a colony-forming unit in culture (CFU-C). Following the development of clonal cell cultures, various *in vitro* assays for early human stem cells have been described, including long-term bone marrow cultures (LTBMC) and the high proliferative potential colony forming cell (HPP-CFU) culture (reviewed by Moore, 1991). Exciting new developments in hemopoietic stem cell research both in preclinical animal models and patients include purification methods for stem cells, characterization of microenvironmental regulators, the use of stem cells as vectors for gene therapy, identification of different new sources of stem cells such as cord blood and HGF-mediated mobilization of stem cells into peripheral blood, and characterization of stem cells at the molecular level (reviewed by Müller-Sieburg et al., 1992)

1.3 Hemopoietic growth factors

The development of cell culture systems for the clonal growth of hemopoietic progenitor cells led to the discovery of a group of soluble polypeptide growth factors which apparently play a fundamental role in the regulation of the blood cell production. In such cultures, individual progenitor cells of a particular hemopoietic lineage are capable of survival, proliferation and terminal differentiation by the virtue of specific regulatory molecules. These mediators have become known as cytokines and were designated according to their effects in a specific bioassay and, usually, their names were abbreviated to appropriate acronyms. Following molecular cloning, it became evident that many of these cytokines possess multiple overlapping activities and have multiple designations. Factors implicated in the modulation of blood cell production include, but are not limited to cytokines such as colony-stimulating factors (CSFs), interleukins (ILs), interferons (IFNs), cachectin or tumor necrosis factor-α (TNF-α),

lymphotoxin or TNF-β, and transforming growth factor β (TGF-β). In addition, several other factors such as prostaglandins E_1 and E_2, lactoferrin, acidic isoferritin, activin, and inhibin have been shown to influence blood cell production. The majority of these cytokines, the hemopoietic growth factors (HGFs), form a family of glycoproteins with specified biological activities and are defined by their ability to support proliferation and differentiation of various blood cell progenitors. In addition to the factors that directly affect these progenitor cell populations, the HGFs include factors that are not growth factors in the classical sense because as single agents these factors do not support colony growth. It was shown that these pleiotropic factors rather act in concert with other HGFs to support hemopoietic cell growth and, furthermore, it was shown that their biological activities are in most cases not limited to the modulation of hemopoiesis. These factors, including IL-1, IL-4, IL-6 and leukemia inhibitory factor (LIF) are capable of inducing the transition of early hemopoietic progenitors into cell cycle and may regulate the expression of receptors of other HGFs. The latter reflects the observed synergistic activities of these factors. Many HGFs display overlapping biological activities on a variety of cell types. Furthermore, the existence of cytokines which inhibit hemopoietic cell proliferation, i.e., TNFs, TGF-β, etc., should be mentioned as well since appropriate regulation of blood cell production results from a complex balance between positive and negative signals. Therefore, analysis of these different classes of factors should not be dissociated. It is conceivable that in physiological settings, HGFs act on the same cell type and modulate their biological actions. Thus, the functional interplay between HGFs acting on the same cell type, either synergistically or antagonistically, will determine the cellular response.

In an attempt to resolve the problem of multiple designations of the HGFs, an international nomenclature has been adopted for many of these cloned cytokines. With respect to the colony-stimulating factors (CSFs), descriptive and more or less historical reasons underlie the designations to refer to these factors. In 1986 at the 6th "International Congress of Immunology", it was decided that the term "Interleukin" would be applied to a given lymphokine only when the complete amino acid sequence was established (Dinarello and Mier, 1987), thus tightening the original definition agreed upon in 1979 (Aarden et al., 1979). As is inevitable in a rapidly developing field, the current nomenclature is not fully rational, since several cloned cytokines such as interferons, tumor necrosis factor and transforming growth factor, produced by lymphoid cells and influencing the growth and/or the differentiation of other lymphoid cells are not given "Interleukin" designations. However, the current nomenclature of CSFs and ILs has been broadly accepted. The CSFs, ILs and some other distinct growth factors are collectively known as hemopoietic growth factors (HGFs).

The biological effects of the HGFs are known to be mediated through interactions with corresponding cell surface receptors present on target cells. The processes upon binding of the HGF to the specific membrane proteins are

largely unknown. Advances in molecular biology facilitated studies to characterize the structures of these cell surface receptors and to elucidate part of the signal transduction mechanism(s). Besides the molecular identification of most of the HGFs, many HGFs receptors have been isolated as well and reports describing mechanisms involved in the signal transduction pathways have recently begun to emerge in the literature. Early events of receptor-mediated growth factor interactions on target cells appear to involve stimulation of tyrosine-, serine-, and threonine-specific phosphorylations and also protein kinase C appears to be activated in some cases. Most of the hitherto identified HGFs and their receptors, the stimulatory activity, the biochemistry and molecular biology, the mode of action as well as the early events triggered by the binding of the HGFs to their receptors will be considered in the next sections (1.3.1-4 and 1.4). Furthermore, the biological activities of the well-characterized HGFs and their major biochemical and genetic features are summarized in Tables 1.1 and 1.2, respectively.

1.3.1 Colony-stimulating factors

The colony-stimulating factors (CSFs) are usually classified by the types of mature cells found in the progeny of specifically stimulated hemopoietic progenitor cells in clonal cell cultures. In the murine system four major types of CSFs were identified by careful analysis of the composition of the colonies grown in the presence of different sources of colony-stimulating activity (CSA). The nomenclature of the colony-stimulating factors resembles the nomenclature of the hemopoietic progenitors by using an identical prefix, i.e., G-CSF stimulates CFU-G to give granulocytic type colonies, M-CSF preferentially stimulates the formation of monocytic colonies. These two CSFs proved to be relatively lineage-specific (Stanley and Heard, 1977; Nicola et al., 1983; Metcalf, 1984; Nicola and Metcalf, 1985; Metcalf, 1986; Clark and Kamen, 1987; Whetton and Dexter, 1989). However, colonies grown in the presence of granulocyte-macrophage-CSF (GM-CSF) or multilineage-colony-stimulating factor (multi-CSF) were found to contain cell types belonging to various hemopoietic lineages. This descriptive nomenclature of the CSFs proved to be ambiguous and even incorrect in some cases. The prefix GM of GM-CSF indicates that this factor stimulates the formation of granulocyte and/or macrophage colonies, which appeared to be only a part of its action on murine hemopoietic cells (Metcalf, 1986). The chosen prefix for multi-CSF seems to be more appropriate to describe its broad range of proliferative and stimulatory effects on hemopoietic cells, including actions on granulocytes, macrophages, erythrocytes, megakaryocytes, eosinophils, mast cells, stem cells and multipotential cells. Due to the multi-action of this CSF as determined by various bioassays, this factor has been designated multiple names abbreviated to appropriate acronyms such as IL-3, interleukin-3; SAF, stem cell activating

Table 1.1 Effects of the major hemopoietic growth factors

Factor[a]	Alternative nomenclature	Cellular source	Hemopoietic activities
Erythro-poietin (Epo)		Kidney, Renal and Hepatic cells	Stimulates proliferation of committed erythroid progenitors; Induces erythroid differentiation and hemoglobin formation; Supports megakaryocyte colony formation
Thrombo-poietin (TPO)	c-Mpl ligand	Liver and Kidney cells	Stimulates megakaryocytopoiesis and the production of platelets; Supports megakaryocyte colony formation
M-CSF	CSF-1	Macrophages Fibroblasts Endothelial cells	Stimulates monocyte/macrophage progenitors; Induces synthesis of other HGFs, including G-CSF, TNF and IFNs by monocytes
G-CSF	CSFβ, Pluripoietin	Macrophages Fibroblasts Endothelial cells Mesothelial cells	Stimulates neutrophilic progenitors, endothelial cell proliferation and functional activation of neutrophilic granulocytes
GM-CSF	CSFα, CSF-2	T lymphocytes Monocytes Fibroblasts Endothelial cells Mesothelial cells Mast cells	Stimulates myeloid progenitor cells; Activates mature neutrophils; Induction of TNF and IL-1; Stimulates platelet production
SCF	c-Kit ligand, KL, MGF, Steel factor	BM stromal cells Fibroblasts Rat liver cells Neurons Macrophages Schwann cells	Synergizes with other HGFs (Epo, GM-CSF, G-CSF, IL-3, IL-6, IL-11) to stimulate myeloid and erythroid colony formation; Stimulates proliferation and differentiation of immature mast cells; Induces histamine release by mast cells
IL-1α/β	Hemopoietin-1, LAF, MCF, Catabolin	Macrophages Endothelial cells Lymphocytes Glial cells Keratinocytes Epithelial cells Neutrophils	Synergizes with other HGFs to support hemopoietic growth; Induces synthesis and release of HGFs, proteases, prostaglandins; Activates T cells by inducing IL-2 production and IL-2 receptor expression; Activates osteoclasts, NK cells and fibroblasts; Induces systemic acute-phase responses
IL-2	TCGF, KHF	T lymphocytes	Stimulates proliferation of activated T cells; Augments natural killer cell activity; Induces synthesis and release of other cytokines; Affects B cell functions and proliferative responses

Factor[a]	Alternative nomenclature	Cellular source	Hemopoietic activities
IL-3	Multi-CSF, SAF, BPA, HCGF, MCGF, PSF	T lymphocytes	Stimulates multipotent hemopoietic stem cells, all myeloid progenitors, pre-B cells, mast cells, NC cells and AML blasts; Supports osteoclasts formation; Increases intracellular histamine levels.
IL-4	BSF-1, BCGF-1 MCGF-2	T lymphocytes Mast cells	Stimulates proliferation and differentiation of B cells; Induces class I and II MHC molecules Stimulates proliferation of T cells and mast cells; Synergizes with CSFs to either stimulate or suppress colony growth; Enhances cytolytic activity of cytotoxic T cells; Induces synthesis of IgE and IgG_1 by B cells
IL-5	TRF, EDF, BCGF-2	T lymphocytes	Stimulates growth and differentiation of B cells and eosinophils; Acts as a T cell replacing factor; Enhances production of IgM, IgG_1, IgG_3 and IgA by B cells
IL-6	BSF-2, HSF, IFN-β2, H-PGF 26 kDa protein	T lymphocytes Fibroblasts	Synergizes with IL-3 and GM-CSF to support colony growth by multipotential hemopoietic progenitor cells;
IL-7	Lymphopoietin 1 (LP-1)	Stromal cells	Stimulates proliferation of pre B cells; Affects fetal-, pro- and mature thymocytes
IL-8	NAF, MONAP, NAP-1, TCF, MDNCF, LAI, LYNAP	Macrophages Lymphocytes Fibroblasts Endothelial cells Chondrocytes	Activates neutrophils; Induces neutrophil chemotaxis; Induces expression of Mac-1 and complement receptor (CR1) on neutrophils
IL-9	P40/TGFIII, MEA	T lymphocytes	Synergizes with IL-3 and GM-CSF to support BFU-E *in vitro* ; Promotes erythroid differentiation in the presence of IL-3 and Epo; Synergizes with IL-3 to stimulate the proliferation of mast cells
IL-10	CSIF	B lymphocytes Monocytes	Inhibits the secretion of pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-8, TNF-α; Enhances IL-2 and IL-4 induced proliferation of thymocytes; Enhances IL-3 and IL-4 induced proliferation of mast cells

Factor[a]	Alternative nomenclature	Cellular source	Hemopoietic activities
IL-11	AGIF	Stromal cells	Supports megakaryocyte colony formation in the presence of IL-3; Increases the number of circulating neutrophils and enhances the generation of platelets *in vivo*; Stimulates erythroid progenitor cells, either alone or in combination with IL-1, SCF, or Epo
IL-12	NKSF CLMF	B lymphocytes Monocytes Mast cells	Stimulates proliferation of activated NK cells; Stimulates early hemopoietic progenitors in the presence of IL-3, SCF and Epo
IL-13	P600	T lymphocytes	Inhibits the expression of pro-inflammatory cytokines (e.g., IL-1,IL-6, IL-8, IL-10 and IL-12); Enhances the expression of surface antigens on B cells; Suppresses cell mediated immunity; Induces the expression of IL-1Rα
IL-14	HMW-BCGF	T lymphocytes	Promotes expansion of normal memory B cells; supports long-term growth of B cells *in vitro*; Inhibits immunoglobulin secretion.
IL-15	IL-T	Epithelial cells Monocytes Kidney cells	Activates NK cells via components of the IL-2 receptor; Induces proliferation and differentiation of activated B cells; Induces IgM, IgG1, and IgA secretion

[a] General nomenclature of the hemopoietic growth factors and their abbreviations are defined in the text. Data are taken from references cited in the text.

factor; BPA, burst promoting activity; HCGF, hemopoietic cell growth factor; MCGF, mast cell growth factor; and PSF, persisting cell-stimulating factor. Erythropoietin (Epo), a plasma glycoprotein which is the primary physiological factor responsible for the generation of erythrocytes (White et al., 1960; Miyake et al., 1977), is generally accepted as a member of the CSFs. Epo-dependent assays have been developed for committed erythroid progenitor cells (Stephenson et al., 1971; Axelrad et al., 1974; Koury et al., 1982) and two major classes of progenitor cells have been defined, termed burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E). BFU-Es are responsive to several other growth factors, including multi-CSF/IL-3 and GM-CSF, but it appears that only Epo is required for their terminal differentiation to (hemoglobinized) erythrocytes. Thus BFU-E are developmentally early, Epo-independent progenitor cells, whereas CFU-E are developmentally late Epo-dependent progenitor cells (Wagemaker, 1985).

Table 1.2 **Basic genetic and biochemical features of the major human hemopoietic growth factors**

Factor[a]	Gene			Protein	
	Chromosome localization	Exons	mRNA (kb)	Mass[b] (kDa)	Human and Mouse cross-reactivity
Erythropoietin	7q21	5	1.6	31-39	cross-reactive
Thrombopoietin	3q27-28	6	1.8[c]	68-85	cross-reactive
M-CSF	1p21	10	4[c] 1.6[c]	35-45 (x2)[d] 20-26 (x2)[d]	human active on mouse, mouse inactive on human
G-CSF	17q11-21	5	2[c]	18-22	cross-reactive
GM-CSF	5q23-32	4	1	14-35	species-specific
SCF	12q22-24	6	6.5[c]	32-45	human inactive on mouse mouse active on human
IL-1α IL-1β	2q 2q	7 7	2.1 1.8	15-17 15-17	cross-reactive cross-reactive
IL-2	4q26-27	4	1	17-23	human active on mouse, mouse inactive on human
IL-3	5q23-32	5	1	15-25	species-specific
IL-4	5q23-31	4	0.6	15-20	species-specific
IL-5	5q31	4	1.6[c]	18-25 (x2)[d]	cross-reactive
IL-6	7p21	5	1.3	21-26	cross-reactive
IL-7	8q12-13	6	1.8[c]	15-25	human active on mouse, mouse inactive on human
IL-8	4q13-31	4	1.8[c]	8-10	cross-reactive
IL-9	5q31.1-31.3	5	0.8	20-30	human inactive on mouse mouse active on human
IL-10	1	5	1.8	17-20 (x2)[d]	human active on mouse mouse inactive on human
IL-11	19q13	5	1.5[c] 2.6[c]	24	cross-reactive

Factor[a]	Gene			Protein	
	Chromosome localization	Exons	mRNA (kb)	Mass[b] (kDa)	Human and Mouse cross-reactivity
IL-12	5q31-33 3q12-13	5 ?	2.3 (p40) 1.3 (p35)	75 (35+40)[e]	species-specific
IL-13	5q31	4	1.3	10	human inactive on mouse mouse active on human
IL-14	?	?	1.8	53	?
IL-15	4q31	8	1.3	15	?

[a] General nomenclature of the hemopoietic growth factors and their abbreviations are defined in the text. Data are taken from references cited in the text.
[b] May vary according to the degree of glycosylation of the polypeptide.
[c] Alternative processing of primary transcript.
[d] Disulfide-linked homodimer composed of two identical subunits.
[e] Disulfide-linked heterodimer composed of two unequal, genetically unrelated subunits.

The genes encoding the murine CSFs have been isolated, i.e., multi-CSF (Fung et al., 1984; Yokota et al., 1984; Miyatake et al., 1985; Campbell et al., 1985; Kindler et al., 1986), GM-CSF (Gough et al., 1984), G-CSF (Tsuchiya et al., 1986), Epo (McDonald et al., 1986). The expression of these genes allowed for large scale production of the murine CSFs which prompted characterization of their biological actions. The biochemistry, molecular biology and biological and physiological functions of the murine CSFs and the cells that produce and respond to murine CSFs have been extensively described and reviewed (Metcalf, 1986) and therefore only murine multi-CSF, generally referred to as murine interleukin-3, will be discussed in section 1.4.

In the human hemopoietic system, a set of five analogous factors has been described, having properties similar to those of the corresponding murine CSFs (Clark and Kamen, 1987; Whetton and Dexter, 1989). Initially two biochemically separable forms of a human CSF, capable of stimulating granulocyte-macrophage colony formation by human cells, were identified (Nicola et al., 1979; Vadas et al., 1984). From their behavior on hydrophobic columns (Abboud et al., 1981) these factors were labeled α (nonbinding) and β (binding). CSFα appeared to be identical to human GM-CSF and CSFβ to human G-CSF. M-CSF, also referred to as CSF-1, found in human urine, was the first human CSF to be purified to homogeneity (Motoyoshi et al., 1978).

Erythropoietin (Epo), the major source being the kidney, has been purified from urine of anemic patients. The purification of human CSFs was hindered by the relatively low amounts present in natural sources and by the lack of reliable biological assays. The isolation of a few milligrams of erythropoietin required more than 2000 liters of urine from severely anemic patients (Miayake et al., 1977), and even then, amino acid sequence information was difficult to obtain. For the same reasons, it took almost ten years before the molecular cloning of human erythropoietin was reported. At the time of the molecular identification of these four human CSFs, little was reported about a human homolog of murine multi-CSF and some investigators even thought that the gene had been lost in evolution and that its function was subsumed by the multipotent CSF activity of human GM-CSF (Cohen et al., 1986; Kindler et al., 1986). Among the five human CSFs, the isolation and identification of human multi-CSF proved to be the most difficult to achieve. Following the molecular cloning of human multi-CSF, as described in chapter 3, it became apparent that the attempts to identify this human sequence were hampered by a very low expression in activated T cells and, moreover, by the low sequence homology with the corresponding murine gene which indicates that the multi-CSF gene has diverged in evolution more rapidly than the genes encoding other HGFs.

The molecular identification and expression of the genes encoding the human CSFs, i.e., M-CSF (Kawasaki et al., 1985; Wong et al., 1987), G-CSF (Nagata et al., 1986; Souza et al., 1986), GM-CSF (Wong et al., 1985), Epo (Shoemaker and Mitsock, 1986) and multi-CSF or IL-3 (Yang et al., 1986; Dorssers et al., 1987, see chapter 3; Otsuka et al., 1988), initiated extensive characterization of their biochemical and biological properties. A structural approach was used to identify the cDNAs for M-CSF and G-CSF as well as erythropoietin. Briefly, the purified natural proteins were submitted to amino acid (aa) analyses, resulting in the aa sequence from which the nucleotide sequence of the respective messenger RNA (mRNA) was predicted. Synthetic oligonucleotides based on these sequences were used as hybridization probes to identify the G-CSF and M-CSF complementary DNAs (cDNAs). Different approaches were applied to isolate human GM-CSF and multi-CSF. Since these CSFs are active at very low (picomolar) concentrations only trace amounts are present in natural sources which made purification to homogeneity extremely difficult. The expression cloning method (see section 3.1) was used to identify human GM-CSF and gibbon IL-3 cDNAs. The latter was used as a hybridization probe to identify the corresponding human gene (Yang et al., 1986). Using another strategy, a human IL-3 cDNA was independently identified at the Department of Radiobiology, Erasmus University, Rotterdam, c/o Radiobiological Institute TNO, Rijswijk (see chapter 3). IL-3 has been recognized as a cytokine with multiple biological actions, including colony-stimulating activities. Therefore, IL-3 is also referred to as multi-CSF. Nevertheless, interleukin-3 is the most widely used designation.

Macrophage-colony stimulating factor (M-CSF)

Human M-CSF is a homodimer glycoprotein consisting of two identical subunits with a molecular weight of 30-40 kDa each. Two related forms of the monomer are produced due to alternative pathways of splicing of the primary M-CSF transcript and differential post translational processing of the polypeptides (Wong et al., 1987). Disulfide bridges join the two monomers to form a biologically active dimeric polypeptide. Human M-CSF has now been shown to be the same molecular entity as CSF-1. The factor is produced by fibroblasts and properly stimulated monocytes and endothelial cells (Sieff et al., 1987a; Horiguchi et al., 1987). M-CSF is a factor that selectively stimulates the proliferation and differentiation of the macrophage lineage and activates mature macrophages (Das and Stanley, 1982). Since human M-CSF is active in mice, no attempts have been made to identify and express the murine M-CSF gene. It is noteworthy that purified mouse M-CSF from natural sources did not support colony formation by human bone marrow progenitor cells (Motoyoshi et al., 1982) and the action of human M-CSF on human cells is weak. Thus, the action of M-CSF on human bone marrow progenitors is controversial. Therefore, it has been suggested that M-CSF has no direct action on human progenitor cells but, rather, stimulates the production of CSFs by adherent accessory cells (Motoyoshi et al., 1982). Based on these observations, i.e., it has been speculated that, during primate evolution, M-CSF has lost its ability to stimulate macrophage progenitor cells but retained the capacity to activate differentiated macrophages (Nicola and Metcalf, 1985). Alternatively, the expression of the M-CSF receptor on the cell surface of bone marrow cells, during primate evolution, has been shifted from immature to more differentiated macrophages. In general , it has been shown that tissue macrophages involved in organogenesis and tissue remodelling have a requirement for M-CSF, whereas macrophages that are involved in inflammatory and immune responses develop independently of M-CSF. It has been established that M-CSF also modulates hemopoietic processes by inducing the release of macrophage cytokines, such as interferon, tumor necrosis factor (TNF), interleukin-1, and G-CSF, as well as inflammatory modulators, such as prostaglandins and plasminogen activator (Sherr, 1990). It has been suggested that, despite its discovery as a HGF, the dominant physiological actions of M-CSF might well be exerted on other cell types such as placental throphoblasts (Sherr, 1990). A large increase in uterine M-CSF level has been observed during pregnancy, suggesting an important role of M-CSF as a modulator of placental development (Bartocci et al., 1986; Pollard et al., 1987).

The M-CSF receptor (M-CSFR) is encoded by the *c-fms* proto-oncogene and is a member of a family of growth factor receptors which exhibits ligand-induced tyrosine-specific protein kinase activity. M-CSFR is a transmembrane glycoprotein with an extracellular domain containing five immunoglobulin-like domains. M-CSF exerts its pleiotropic effects by binding to this single type of

high affinity cell surface receptor. Initially, it was thought that the single genes for M-CSF and its receptor are both located on the long arm of human chromosome 5 and map to the 5q33.1 and 5q33.3 band, respectively (Sherr, 1990). Recently, it was shown that the single copy M-CSF gene is localized at human chromosome 1p13-p21 and not at 5q33.1 (Landegent et al., 1992).

Granulocyte-colony stimulating factor (G-CSF)

Human G-CSF is a glycoprotein of 174 amino acids (aa) with a molecular weight of approximately 25 kDa (Souza et al., 1986) that requires internal disulfide bridges for its biological activity. Human G-CSF, CSF-β and pluripoietin appeared to be the same molecule. G-CSF can be produced by macrophages (Rambaldi et al., 1987), fibroblasts (Rennick et al., 1987a) and endothelial cells (Sieff et al., 1987a) in response to specific stimuli. The species cross-reactivity of human G-CSF is reflected by a high sequence homology at the DNA level as well as at the protein level, receptor binding cross-reactivity to human and murine cells and similar biological response in several species (Nagata et al., 1986). Although it has been accepted that G-CSF exhibits a relatively restricted lineage specificity, affecting mainly the neutrophilic lineage, *in vitro* studies show that it might also affect multipotential hemopoietic progenitors (Ikebuchi et al., 1988). Furthermore, G-CSF stimulates neutrophilic phagocytosis, superoxide generation, antibody-dependent cellular cytotoxicity (ADCC), and the binding of chemotactic molecule receptors (Wang et al., 1988).The human gene encoding G-CSF has been mapped to chromosome 17 near the centromere (Laver and Moore, 1989).

G-CSF exerts its biological effects through binding to specific cell surface receptors. A single class of high affinity receptors is expressed on both human and murine hemopoietic progenitors, neutrophils, and a variety of non-hemopoietic cells. The gene encoding the receptor for human G-CSF (G-CSFR) has been identified (Larsen et al., 1990). The G-CSFR has a single transmembrane domain. The extracellular domain of the G-CSFR contains an N-terminal immunoglobulin domain, a structural motif (tryptophan-serine-x-tryptophan-serine [WSXWS]) characteristic of the hematopoietin or cytokine receptor superfamily (Bazan, 1990), and three tandem repeats of the fibronectin type III domain. The G-CSFR does not contain intracellular kinase domains. It was shown that upon G-CSF stimulation, the JAK/STAT pathway of signal transduction is activated (Ihle et al., 1994). Thus, binding of a HGF ligand to its receptor stimulates the physical association of the membrane-proximal region of the ligand-bound receptor complex with members of the Janus kinase (JAK) family of cytoplasmic protein tyrosine kinases. Subsequently, the JAK kinases become tyrosine phosphorylated and activated. The activated JAKs can phosphorylate a family of transcription factors called the signal transducers and activators of transcription (STATs). This novel signalling pathway that is shared by all members of the hematopoietin or cytokine receptor superfamily is

discussed in more detail elsewhere (see section 1.4.3; reviewed by Ihle et al., 1994; Ihle and Kerr, 1995). With respect to G-CSF signal transduction, it was shown that the tyrosine kinase JAK1 is involved which in turn activates the STAT3 protein.(Nicholson et al., 1994; Tian et al., 1994). It has been established that the cytoplasmic domain of the G-CSFR contains distinct functional subdomains that are coupled to specific receptor signaling activities (Dong et al., 1993). The membrane-proximal region of 55 aa is sufficient for the transduction of proliferative signals. The further downstream sequence of 30 aa significantly enhances mitogenic signaling. In contrast, the distal carboxy-terminal region of the G-CSFR couples to differentiation since this region was shown to be essential for mediating signals for terminal granulocytic maturation, whereas it inhibits mitogenic signalling.

Granulocyte/macrophage-colony stimulating factor (GM-CSF)
Human GM-CSF is a heterogeneously glycosylated protein of 127 aa that has a molecular weight of 14.5 kDa when non-glycosylated and appeared to be identical to CSF-2 and CSFα. The GM-CSF gene encodes a 144 aa precursor polypeptide which is processed at the amino terminus by the removal of 17 residues and the mature protein is subsequently glycosylated at the two potential N-linked glycosylation sites (carbohydrates attached through the amino group of asparagine residues). The natural GM-CSF molecule is highly glycosylated, some forms contain more than 50 % carbohydrate depending on the source it was derived from. Surprisingly, deglycosylated GM-CSF showed increased biological activity *in vitro* (Moonen et al., 1987) and also an increased affinity for its cell surface receptor (Chiba et al., 1990). However, *in vivo* studies showed that less heavily glycosylated GM-CSF molecules are more rapidly cleared from the bloodstream. Thus, unglycosylated GM-CSF showed a decreased stimulation of the number of circulating blood cells, as compared to highly glycosylated GM-CSF (Donahue et al., 1986). There is 56 % aa sequence homology between human and murine GM-CSF and their corresponding DNA sequences display 70 % homology. Nevertheless, the human and murine GM-CSF proteins are species-specific. The gene encoding human GM-CSF is located on chromosome 5 and has been mapped to the 5q23-q32 band within a cluster of other HGF genes (Yang et al., 1988). Initially, recombinant as well as purified human GM-CSF was shown to stimulate *in vitro* the development of colonies consisting of neutrophilic granulocytes, granulocyte/macrophages, macrophages or eosinophilic granulocytes (Sieff et al., 1985). In addition, GM-CSF co-operates with erythropoietin to enhance the formation of erythroid colonies demonstrating that GM-CSF has burst-promoting activity. Using highly purified human bone marrow progenitors, it has been demonstrated that GM-CSF stimulates selectively BFU-E, CFU-GEMM and, predominantly, CFU-Eo, but not CFU-GM, CFU-M or CFU-G (Bot et al., 1989). Apparently, GM-CSF can only stimulate significant numbers of CFU-GM, CFU-G, and CFU-M in the

presence of monocytic cells capable of producing auxiliary HGFs. In addition to the effects on normal bone marrow cells, GM-CSF acts on several human leukemic cell lines such as HL-60 and KG-1 (Tomonoga et al., 1986) either by stimulating proliferation or by inducing differentiation. Furthermore, colony formation of acute myeloblastic leukemia (AML) cells in response to recombinant human GM-CSF has been reported (Griffin et al., 1986; Vellenga et al., 1987; Delwel et al., 1988). Continuous administration of recombinant human GM-CSF to rhesus monkeys (*Macaca mulatta*) resulted in a substantial leukocytosis and a significant elevation in thrombocyte counts (Donahue et al., 1986; Wielenga, 1990). In addition, administration of human GM-CSF following autologous bone marrow transplantation in rhesus monkeys resulted in a dose-dependent rise in peripheral blood cell counts (Wielenga, 1990).

GM-CSF mediates its pleiotropic effects through binding to specific cell surface receptors. The GM-CSF high affinity receptor complex is composed of two components, i.e., the α subunit (GM-CSFRα) that exhibits low affinity GM-CSF binding and a β chain (GM-CSFRβ) that lacks direct GM-CSF binding activity. The α and β subunits of the human GM-CSF receptor have been isolated by expression cloning (Gearing et al., 1989; Kitamura et al., 1991; Kitamura and Miyajima, 1992). It was shown that this β subunit of the heterodimeric GM-CSF receptor is also a component of the IL-3 and IL-5 receptors (see also section 1.4.3 and reviews by Nicola and Metcalf, 1991; Miyajima, 1992; Miyajima et al., 1993; Goodall et al., 1993). The Ras signaling pathway as well as the JAK/STAT pathway of signal transduction are thought to play critical roles in differential GM-CSF mediated signaling (reviews by Ihle et al., 1994; Ihle and Kerr, 1995).

Stem cell factor (SCF)

In addition to the CSFs, a novel pleiotropic cytokine with growth factor activities, generally called stem cell factor (SCF) but also termed c-Kit ligand (KL), mast cell growth factor (MCFG) or steel factor (SLF) has been identified (Zsebo et al., 1990a,b; Williams et al., 1990; Martin et al., 1990). This growth factor encoded by the steel locus (*sl*) in mice is the ligand for the tyrosine kinase-associated receptor encoded by the c-kit proto-oncogene at the dominant white spotting (*W*) locus (Zsebo et al., 1990; Huang et al., 1990). Mice harboring mutations at the *sl* and *W* loci express similar pleiotropic abnormalities, including severe deficiencies in hemopoiesis (reviewed by Russel, 1979). Apparently, the SCF-triggered c-kit signal transduction pathway is essential for early hemopoiesis *in vivo*. Cloning data have demonstrated that SCF can exist in either a membrane-bound form or a soluble, secreted form (Anderson et al., 1990), both of which are derived from one large SCF transcript of approximately 6.5 kb. Both soluble and transmembrane forms of SCF have growth factor activities. Human and mouse SCF cDNA sequences encode a transmembrane protein (45 kDa) composed of a 25 aa signal peptide, a

189 aa extracellular domain, a 23 aa hydrophobic transmembrane span, and a 36 aa cytoplasmic segment. An alternative splice variant in which a complete exon was deleted resulted in a transmembrane molecule 28 aa shorter in the extracellular domain. Both the larger 45 kDa form (KL-1) and the smaller 32 kDa form (KL-2) are cleaved to produce soluble factors of 31 kDa and 23 kDa, respectively (Huang et al., 1992). As yet, it is unclear what the roles of soluble versus membrane-bound SCF may be. The membrane form has been suggested to be particularly important in mediating the overall process of cell proliferation and in mediating cell adhesion and interaction (Godin et al., 1991; Huang et al., 1992). SCF has several N- and O-linked glycosylation sites and contains four cysteine residues which are all four involved in intramolecular disulfide bonds (Lu et al 1991). Native soluble SCF is heavily glycosylated and exists as a non-covalently associated homodimer in solution. Non-glycosylated recombinant human soluble SCF is biologically active in *in vitro* bioassays, suggesting that glycosylation is not required for bioactivity *in vivo*. As alluded to earlier, SCF exerts its biological activities by binding to the tyrosine kinase-associated receptor encoded by the c-kit proto-oncogene. The human gene encoding SCF has been mapped to chromosome 12 at band q22-24 (Anderson et al., 1991).

Major biological activities ascribed to SCF include growth factor activities on diverse hemopoietic target cells indicating its important role in hemopoiesis (Anderson et al., 1990; Martin et al., 1990; Zsebo et al., 1990b; Ogawa et al., 1991; Tsai et al., 1991a,b; Brandt et al., 1992; Rennick et al., 1995), a neurotrophic role as promoter of the survival and maturation of sensory neurons (Carnahan et al., 1994), and a role in the promotion of survival and/or proliferation of primordial germ cells (Godin et al., 1991). With respect to the hemopoietic activities, SCF seems to play an essential role in early blood cell formation by acting in a synergistic manner with various HGFs (IL-3, IL-6, IL-11, GM-CSF, G-CSF and Epo) on primitive precursor cells to induce early myelopoiesis and early erythropoiesis (McNiece et al., 1991; Briddell et al., 1993). In addition, recombinant rat SCF accelerates the recovery of bone marrow function following BM transplantation in mice by expanding the primitive cellular components of the BM, including pre-CFU-S, BFU-E, CFU-GEMM, CFU-Meg and CFU-GM (Tong et al., 1993). SCF is a potent stimulator of the proliferation of mature and immature mast cells and also affects the maturation of immature mast cells (Tsai et al., 1991a,b; Rennick et al., 1995). In combination with IL-7, SCF promotes early B cell progenitors and in combination with IL-2 and IL-7, it stimulates proliferation of thymocytes. A rather peculiar species specificity was observed for human SCF which shows approximately 79 % similarity at the protein level to murine and rat soluble SCF. Whereas both rat and mouse SCF are fully active on human cells, the human protein is 800 fold less active on mouse and rat cells (Martin et al., 1990).

Erythropoietin (Epo)

Human Epo is a glycoprotein of 166 residues with a molecular weight of 31 kDa and is encoded by a gene which is mapped to chromosome 7 (Jacobs et al., 1985). The carbohydrate content is approximately 40 % and consists of one O-linked and three N-linked oligosaccharides. The *in vivo* biological activity of erythropoietin is completely dependent on the presence of sialic acid (Lowry et al., 1960) since asialoerythropoietin is rapidly cleared from the circulation by hepatic cells (Goldwasser et al., 1974). The presence and structure of the carbohydrates (Lai et al., 1986; Fukuda et al., 1989) appeared to be important for biological activity *in vivo* contrasting the observed enhanced activity of asialoerythropoietin *in vitro* (Goldwasser et al., 1974). Amino acid sequence homology between mouse and human Epo is significantly higher than found for other CSFs and Epo appeared to be species cross-reactive. The mature Epo proteins share 81 % homology and large regions of the primary structure have remained completely conserved. Epo is synthesized in the kidney and circulates in the blood, increasing the number of developing erythroid precursors and accelerates the release of erythrocytes from the bone marrow. Immature erythroid progenitors are responsive to several other HGFs, including GM-CSF and IL-3, but it appears that only Epo is required for their terminal differentiation (Suda et al., 1986).

Epo exerts its biological effects through binding to specific receptors. A gene encoding the human Epo cell surface receptor (EpoR) has been identified and cDNA analysis predicts that the encoded transmembrane protein contains the WSXWS motif and a set of four conserved cysteine residues (Winkelman et al., 1990). The EpoR, together with receptors for IL-2, IL-3, IL-4, IL-5, IL-7, and GM-CSF, belong to the hematopoietin or cytokine receptor superfamily which is characterized by homology at the N-terminal region of the extracellular ligand-binding domain, conserved cysteine residues, and a WSXWS motif (Bazan, 1990). In contrast to the IL-2R, the EpoR consists of a single component that is capable of ligand-binding and signal transduction. Apparently, the high affinity EpoR does not require the coexpression of different subunits as was shown for IL-2, IL-3, IL-5, and GM-CSF (Nicola and Metcalf, 1991; Voss et al., 1994). The mechanism of signal transduction by the Epo receptor complex is not yet fully understood. EpoR homodimerization is considered to be a first step in the signal transduction process (reviews Ihle et al., 1994; Ihle and Kerr, 1995). Dimerization is associated with increased activity for JAK2. Recruitment of JAK2 to the oligomerized receptor results in autophosphorylation of JAK2 which is associated with activation of JAK2 kinase activity. The activated JAK2 phosphorylate the receptor as well as cellular substrates (e.g. STAT5) that are recruited to the receptor-kinase complex. This sequence of events (see above) following Epo binding has been proposed by Ihle et al. (1994).

Thrombopoietin (TPO)

The unequivocal identification of TPO, the candidate regulator of circulating platelet levels, has proved to be a daunting task. This growth factor is thought to be a lineage-specific cytokine affecting the proliferation and maturation of committed cells resulting in the production of megakaryocytes and platelets. Four other hemopoietic regulators with proliferative actions on megakaryocytes (IL-3, IL-6, IL-11 and leukemia inhibitory factor) were molecularly cloned and their biological characterization proved that these factors have some abilities to increase platelet levels (reviewed by Metcalf, 1994). Finally, cDNAs for both human and murine TPO have been cloned and their encoded glycoproteins showed selective actions on megakaryocytes (Lok et al., 1994; Kaushansky et al., 1994; Wendling et al., 1994; De Sauvage et al., 1994; Bartley et al., 1994). Cloning data demonstrated that thrombopoietin is the ligand for the c-Mpl receptor encoded by the c-*mpl* proto-oncogene (De Sauvage et al., 1994). This proto-oncogene is a newly described member of the hemopoietin receptor superfamily and its function has been implicated in megakaryocytopoiesis (Methia et al., 1993). The human thrombopoietin gene encodes a mature protein of 332 aa that can be divided into two domains: an amino terminal half of 153 aa with homology to erythropoietin and a unique C-terminal domain of 179 aa containing multiple potential N-linked glycosylation sites (Gurney et al., 1995). The predicted molecular weight of the mature thrombopoietin nonglycosylated protein is 38 kDa. However, immunoprecipitation of human TPO shows a protein of 68-85 kDa, suggesting that the molecule is highly glycosylated. Species comparison showed that the erythropoietin-like domain is highly conserved, whereas the C-terminal domain is less conserved. Two splice forms, one alternative splice form involving a deletion in exon 6, have been identified in humans, pigs and mice. Although both splices forms are highly conserved, the alternative splice form encoding a four aa deletion was shown to be biologically inactive. The physiological role of this alternative splice form is not clear. Both recombinant murine and human TPO were able to activate the murine and human c-Mpl receptors, indicating an absence of strict species specificity. The human gene is localized to the long arm of chromosome 3 at band 3q27-28 (Gurney et al., 1995).

The biological activities of TPO are restricted to megakaryocytopoiesis. Recombinant murine TPO showed powerful actions in increasing platelet numbers in mice (Lok et al., 1994). In addition, recombinant human thrombopoietin stimulated human megakaryocytopoiesis *in vitro* alone or in the presence of other exogenously added early-acting HGFs (De Sauvage et al., 1994; Bartley et al., 1994; Kaushansky et al., 1994; Wendling et al., 1994). Experiments using antisense oligonucleotides to c-*mpl*, the proto-oncogene encoding the receptor for TPO, provided evidence that this receptor and its putative ligand are involved in regulating megakaryocytopoiesis (Methia et al., 1993). These observations were further substantiated using c-*mpl* deficient mice

generated by gene targeting (Gurney et al., 1994). The c-*mpl* deficient mice showed severe thrombocytopenia, i.e., 85 percent decrease in platelet and megakaryocyte numbers, whereas the number of other hemopoietic cell types were not affected. These mice also had increased levels of circulating TPO, indicating that in response to thrombocytopenia, TPO levels are up-regulated. These studies demonstrated that thrombopoietin is a humoral regulator of megakaryocytopoiesis and thrombopoiesis and acts through binding to the c-Mpl receptor.

1.3.2 Interleukins

Interleukins are soluble cytokines which were originally thought to be exclusively produced by macrophages and activated T or B cells, and to act primarily on B or T lymphoid cells. Initially, these factors were designated according to their activities in bioassays and their names were abbreviated to appropriate acronyms, i.e., LAF, lymphocyte activating factor (now IL-1); TCGF, T cell growth factor (now IL-2); BCDF, B cell differentiating factor (now IL-6), etc (Harrison and Campbell, 1988). Following the purification and molecular identification of several cytokines, the descriptive nomenclature appeared to be unsatisfactory since activities exerted by the same molecule were designated multiple names. In addition, identical acronyms were invented for distinct factors. The interleukin nomenclature (Aarden et al., 1979; Dinarello et al., 1987) adopted for cloned cytokines has now been applied for IL-1 to IL-9. However, some cloned cytokines, i.e., the interferons (IFNs) and the colony-stimulating factors (CSFs), retain their original names for historical reasons. In the next paragraphs IL-1 to IL-15 are discussed.

Interleukin-1 (IL-1)
IL-1 was initially defined as a lymphocyte-activating factor (LAF) and has also been termed endogenous pyrogen (EP), mononuclear cell factor (MCF), and catabolin. Purified protein preparations which displayed characteristic IL-1 activities showed different isolelectric points a well as distinct molecular weights. These observations raised the question as to whether the array of biological activities might reside in a single precursor protein which is differentially processed, leading to the appearance of active polypeptides with different biochemical features, or that the activities characteristic of IL-1 were shared by several different proteins. The initial isolation and identification of a murine (Lomedico et al., 1984) as well as a human cDNA (Auron et al., 1984) encoding the LAF activity did not clarify this issue. This intriguing question was resolved following the molecular cloning of two distinct cDNAs encoding proteins with a characteristic IL-1 activity termed IL-1α and IL-1β (March et al., 1985). The genes encoding both classes of human IL-1 were mapped to chromosome 2. Both factors have a molecular weight of 17 kDa but seem to be

synthesized as larger precursors, with predicted primary translation products of approximately 30 kDa. These precursor proteins were shown to be proteolytically processed into smaller active forms, thereby, resolving the inconsistency of the variable reported molecular weights. In contrast to most of the HGFs, neither form of IL-1 contains a signal sequence and, therefore, the mechanism by which cells release IL-1 remains to be determined. The aa sequences of mature IL-1α and IL-1β are well conserved among various species during mammalian evolution (IL-1α: 60-70 %; IL-1β: 75-78 %). Both proteins have multiple glycosylation sites, but non-glycosylated recombinant factors have biological activities similar to naturally occurring forms of the molecules.

IL-1 is produced by a variety of cell types and has multiple biological activities *in vivo* and *in vitro*, including functioning as an immunological and inflammatory mediator (Dinarello et al., 1982; Luger et al., 1983), initiating a cascade of events leading to the production of other HGFs (Lee et al., 1987; Rennick et al., 1987a; Fibbe et al., 1988), and activating mature blood cells (Pike and Nossal, 1985). IL-1 was also shown to act upon early hemopoietic progenitors (Stanley et al., 1986) termed high proliferative potential colony forming cells (HPP-CFC). Colony formation was not supported by IL-1 as a single agent but required the presence of other factors, such as IL-3, GM-CSF, G-CSF or M-CSF which apparently interact with IL-1 (Moore and Warren, 1987; Mochizuki et al., 1987). However, it has been reported that IL-1, in contrast to IL-6, did not enhance the effect of IL-3 on colony formation of highly purified human bone marrow progenitors (Ikebuchi et al., 1987; Leary et al., 1988). In these experimental settings, HGFs may induce the production of other HGFs in distinct cell types which makes it difficult to distinguish direct from indirect effects. In response to IL-1, bone marrow stromal cells were shown to produce large amounts of IL-6. Furthermore, IL-1 induces the production of GM-CSF, G-CSF, M-CSF, IL-3, and other cytokines (reviewed by Dinarello, 1991). These results indeed suggest that IL-1 primarily affects hemopoiesis indirectly by inducing growth factor production in both accessory cells and stromal cells.

The existence of a natural IL-1 receptor antagonist (IL-1ra) that binds to the IL-1 receptor but has no IL-1-like signal transducing activity has been reported (Hannum et al., 1990). The fact that the IL-1ra is produced by the same cells that produce IL-1 (Eisenberg et al., 1990) and the receptor binding specificity warrant the conclusion that this receptor antagonist is a physiologically significant regulator of IL-1. Upon identification of the protein, the cDNA for the IL-1ra has been cloned and expressed for further characterization. It was shown that IL-1ra molecules compete with IL-1α and IL-1β for binding to the IL-1 receptor indicating that this IL-1ra may inhibit IL-1 activities. Further binding studies are needed to determine the physiological role of both the agonist and the antagonist which may shed some light on the regulatory mechanism(s) of IL-1.

IL-1α and IL-1β exert their effects by binding to specific receptors. The molecular cloning of a murine IL-1 receptor cDNA revealed that the IL-1 receptor, an 80 kDa glycoprotein, shows similarities to the immunoglobulin gene superfamily (Sims et al., 1988). The intracellular domain does not possess a tyrosine kinase sequence, but a kinase C related acceptor site which may be involved in the activation of the IL-1 receptor. A second type of IL-1 receptor (68 kDa) has been identified (Dinarello, 1991). Thus, two distinct receptors (IL-1R Type I and IL-1R Type II), each binding both forms of IL-1 have been identified (Sims et al., 1993). The two IL-1 receptors show low sequence homology and have a cytoplasmic domain that differ significantly. It has been suggested that only the IL-1 receptor Type I is capable of transducing a signal following ligand binding and that all of the biological effects attributed to IL-1 are mediated by this receptor (Sims et al., 1993). Type II IL-1 receptor may serve as a decoy for IL-1, thus antagonizing and regulating IL-1 activities. Alternatively, the function of the membrane-bound Type II receptor is to serve as the precursor for a soluble IL-1 binding factor that can be shed under appropriate circumstances to antagonize and modulate IL-1 activity (Sims et al., 1993). However, the precise function of Type II IL-1 receptor is not known.

Interleukin-2 (IL-2)

IL-2, a 23 kDa glycoprotein, was originally identified as a factor, produced by activated T helper cells, that stimulates the production of cytotoxic T cells and natural killer cells. Therefore, the factor was initially termed T-cell growth factor (TCGF). However, many other names and acronyms describing the biological actions of this factor have been given to this factor. The cDNAs for murine (Kashima et al., 1985) and human IL-2 (Taniguchi et al., 1983) have been identified and the gene encoding IL-2 comprises four exons. The human gene is located on chromosome 4 and encodes a precursor polypeptide of 153 aa from which 20 residues are functioning as a signal peptide. There are some similarities in genomic organization of IL-2, IL-4, IL-5 and GM-CSF suggesting the presence of related structural and functional domains (Smith, 1988).

With respect to its molecular weight and binding characteristics, the structure of the IL-2 receptor has been a paradox for a long time which was partially resolved by the finding that the presence of two distinct polypeptides is necessary for the formation of a high affinity receptor complex (Teshigawara et al., 1987; Tsudo et al., 1986). For several years the high affinity IL-2 receptor capable of transducing the IL-2 signal was thought to consists of an alpha chain (IL-2Rα) and a beta chain (IL-2Rβ). Eventually, it was discovered that a third chain, IL-2Rγ, was necessary for high affinity binding, ligand internalization and signalling. Thus, the biological activities of IL-2 are mediated by the binding of the factor to a multimolecular receptor. A current model of this IL-2 receptor complex (an αβγ trimer), in which all three chains of the receptor are

in contact with the ligand, has been described (Voss et al., 1994). As yet, the IL-2 receptor is the only known HGF receptor that is comprised of three polypeptide chains. Both IL-2Rβ and IL-2Rγ are members of the hematopoietin or cytokine receptor superfamily (Bazan, 1990), whereas IL-2Rα is not a member of any described family. Besides the αβγ multimeric complex, the presence of αβ and βγ heterodimeric complexes was shown as well. The physiological role of these different IL-2R complexes and their role in transduction of the IL-2 signal is not entirely clear. The IL-2Rβ does not contain a consensus sequence for the ATP binding site of tyrosine kinase (Voss et al., 1994). Therefore, other phosphorylation mechanisms must play a critical role in IL-2 signaling. The JAK kinase system seems to be a potential candidate since JAK2 and JAK3 were shown to be physically associated with IL-2R complexes (Miyazaki et al., 1994; Ihle et al., 1994).

IL-2 appears to play an important role in the immune response (Robb, 1984) by controlling T cell proliferation and activating the T cell-mediated secretion of several other lymphokines (Howard et al., 1983), and by activation of natural killer cells (Grimm et al., 1982). With regard to the IL-2-induced T cell proliferation, a few intriguing observations (reviewed by Robb, 1984; Touw, 1986) are noteworthy such as: i) T cells proliferate in response to T cell receptor antigens resulting in enhanced expression of the high-affinity IL-2 receptor, ii) IL-2 receptor is internalized after interaction with IL-2, and iii) the physiological signal for IL-2 production by T cells is activation through antigen binding to the T cell receptor. Although the mechanism is not entirely understood, it has been recognized that antigen stimulation of the T cell antigen receptor coordinates the transcriptional activation of the genes encoding IL-2 as well as its receptor. Thus, IL-2 is an autocrine factor, driving the expansion of the antigen-specific cells. In addition, IL-2 acts as a paracrine factor, influencing the activity of other cells.

Interleukin-3 (IL-3)

The genetic, biochemical, biological and physiological features of murine IL-3 will be described in section 1.4. The molecular cloning of human and rhesus monkey IL-3 and their biological characterization will be presented and discussed in chapter 3, 4 and 5 respectively. Identification and characterization of the IL-3 coding sequences of chimpanzee, tamarin and marmoset will be presented in chapter 6. Structure-function relationship and the molecular evolution of IL-3 will be discussed in chapter 6 as well.

Interleukin-4 (IL-4)

IL-4 was initially described as a T cell-derived lymphokine which stimulates proliferation of resting B cells. It was originally referred to as B cell stimulatory factor 1 (BSF-1). Murine IL-4 cDNA (Noma et al., 1986; Lee et al., 1986) and the human homolog (Yokota et al., 1986) have been isolated and

share about 70 % DNA sequence homology and 50% homology at the aa level. In spite of this considerable homology, the mouse and human IL-4 proteins are not cross-reactive. The human IL-4 genomic sequences were localized to chromosome 5q23-31 by in situ hybridization and by somatic cell hybrid analysis (Yokota et al., 1988). The human and mouse IL-4 gene have been mapped to a cluster of HGF genes encoding IL-3, IL-5, IL-9, IL-13 and GM-CSF at human chromosome 5 and mouse chromosome 11, respectively (Van Leeuwen et al., 1989). Cleavage of the human IL-4 precursor yields a mature protein of 128 aa with a predicted molecular weight of 14.9 kDa. Both human and mouse IL-4 proteins have multiple potential glycosylation sites and contain 6 cysteine residues which are thought, by analogy with the murine molecule, to form three disulfide bonds (Paul and Ohara, 1987). Native as well as recombinant human IL-4 produced by yeasts are hyperglycosylated and have a molecular-mass range of 45-95 kDa, which could be reduced to a single band of about 15 kDa by treatment with N-glycanase. The hyperglycosylated and deglycosylated forms of IL-4 have comparable biological activities (Solari et al., 1989).

IL-4 is an anti-inflammatory cytokine that exhibits multiple immunomodulatory functions on a variety of cell types, including T cells, B cells, monocytes, neutrophils, hemopoietic progenitors, fibroblasts, endothelial and epithelial cells (reviewed by Paul and Ohara, 1987; Kishi et al., 1989; Banchereau and Rybak, 1994). IL-4 is known to be a potent mediator of proliferation and differentiation of hemopoietic progenitors in several lineages. Studies on the synergy between IL-4 and other cytokines reveal that IL-4 enhances the formation of granulocyte and erythrocyte colonies in the presence of G-CSF and erythropoietin , respectively (Rennick et al., 1987b; Peschel et al., 1987). It has been demonstrated that IL-4 can enhance or antagonize the growth factor-dependent proliferation of hemopoietic progenitor cells, including IL-3 dependent cells (Rennick et al., 1987b). IL-4 enhances the IL-5 mediated IgA production in LPS-stimulated B cell cultures (Coffman et al., 1987) indicating functional interplay of HGFs in immuno-inflammatory responses. The dramatic inhibitory effect of IL-4 mediated IgE production by IFN-γ is one of several examples of antagonizing effects of HGFs on one cell type (Coffman and Carty, 1986).

The biological activities of IL-4 are mediated by specific cell surface receptors, expressed on a variety of cell types (Park et al., 1987; Paul and Ohara, 1987). Cross-linking experiments with recombinant IL-4 allowed the characterization of a putative IL-4 receptor with a molecular weight of 138-145 kDa (Park et al., 1988). Different forms of IL-4 receptors, resulting from alternative splicing, have been identified following molecular cloning of the gene encoding the IL-4 cell surface receptor (Mosley et al., 1989). All forms showed IL-4 binding properties. However, the functional IL-4 receptor appeared to be a heterodimeric molecule consisting of a ligand-binding subunit

(IL-4R) and a common γ chain (γ_c), originally identified as a component of the IL-2 receptor complex and now shown to be involved in various HGF receptor complexes, i.e., IL-2, IL-4, IL-9, IL-13 and IL-15 (Banchereau and Rybak, 1994). The presence of γ_c increases the IL-4 affinity for the receptor, but more important, the dimerization of the two components (IL-4R and γ_c) of the IL-4 receptor is required for signalling (Banchereau and Rybak, 1994). The IL-4 signaling pathway involves the activation of JAK1 and JAK3 and a STAT-like protein (Ihle et al., 1994; Witthuhn et al., 1994). An intriguing form of IL-4 cDNA isolated from mouse cells is the one that encodes a soluble, not membrane bound, form of the IL-4 receptor (Mosley et al., 1989). The physiological nature of the secreted form of IL-4 receptor is not yet understood. However, the soluble form has been shown to neutralize the growth-promoting activity of IL-4 (Mosley et al., 1989) suggesting an important role as a physiological regulator of IL-4 function.

Interleukin-5 (IL-5)

IL-5 has multiple effects on hemopoietic cells and has been characterized as an eosinophil differentiation and activating factor. IL-5 appeared to be functionally related to IL-4. Both factors stimulate the proliferation and differentiation of B lymphocytes (Cosman, 1988) and overlap in additional biological actions as well, but are biochemically distinct factors. The activities induced by T cell-replacing factor (TRF), B cell growth factor II (BCGF-II) and eosinophil differentiation factor (EDF) have all shown to be attributed to IL-5. The multiple effects of IL-5 include support of B cell growth and differentiation, mediation of T cell differentiation, induction of IL-2R expression on B cells, enhancement of IgM, IgG_1, IgG_3 and IgA production by non-LPS stimulated B cells, as well as IgA production by LPS stimulated B cells and support of eosinophil growth and differentiation (Swain et al., 1988). The effects of IL-5 on eosinophils (Campbell et al., 1987) suggests a critical role for this molecule in diseases caused by allergens and parasites. The complete nucleotide sequences of the mouse and human IL-5 cDNAs (Kinashi et al., 1986; Azuma et al., 1986) have been determined and display 77 % homology. The activity of human and murine IL-5 appeared to be cross-reactive (Campbell et al., 1987). The human IL-5 gene is mapped to chromosome 5q23.3 to 5q32 (Sutherland et al., 1988).

IL-5 exerts its diverse biological effects on target cells by binding to specific cell surface receptors. The high affinity receptor complex, capable of transducing the IL-5 signal, is composed of two membrane proteins, an alpha chain (IL-5Rα) and a beta chain (IL-5Rβ). The human IL-5Rβ subunit has been found to be identical to the β subunit of the human GM-CSF high affinity receptor complex, as well as to the β subunit of the human high affinity IL-3 receptor complex (Nicola and Metcalf, 1991, Kitamura et al., 1991; Miyajima, 1992; Miyajima et al., 1993; Goodall et al., 1993). The IL-5Rβ subunit is supposed to play a pivotal role in IL-5 signaling. Recently, it was shown that IL-

5 signaling in human eosinophils involves the JAK/STAT pathway of signal transduction. Upon IL-5 binding to its receptor, JAK2 was readily phosphorylated and subsequently STAT1α was shown to be activated (Van der Bruggen et al., 1995).

Interleukin-6 (IL-6)

IL-6 was originally described as a T cell-derived factor that induced terminal differentiation of activated B cells to Ig producing cells. Molecular identification of the B cell stimulatory factor-2 (BSF-2) cDNA (Hirano et al., 1986; Zilberstein et al., 1986; Brakenhoff et al., 1987) showed that this molecule is identical to hybridoma-plasmacytoma growth factor (H-PGF), hepatocyte stimulating factor (HSF), interferon-β 2 (IFN-β 2), and an 26 kDa protein (P26). The primary human protein sequence deduced from the cDNA reveals that IL-6 consists of 184 aa with two potential N-linked glycosylation sites and four cysteine residues (Hirano et al., 1986). The IL-6 protein shows a significant homology with G-CSF and similarity in the tertiary structure (Hirano et al., 1986). The DNA coding sequence of rat IL-6 is 92 % identical to the murine homologous sequence and 68 % identical with the corresponding human sequence, whereas the degrees of protein identity are 93 and 58 %, respectively (Northemann et al., 1989). Although the degree of sequence conservation between rodents and man is surprisingly low compared to the conservation within the rodent species, IL-6 acts across species boundaries. The IL-6 gene is located on the short arm of human chromosome 7. Human IL-6 appears to be both N- and O-glycosylated, whereas the murine and rat IL-6 sequences lack potential N-linked glycosylation sites. As to whether any of the multiple functional properties of human IL-6 are specifically dependent on glycosylation or whether murine and rat IL-6 lack those properties is as yet unknown. Several transcriptional control elements (NF-IL6; NF-κB; serum responsive elements and AP-1 binding sites) have been identified in the 5'-flanking region of the IL-6 gene (Brakenhof, 1990). The regulation of IL-6 gene expression is rather complicated and include differential transcription initiation (Yasukawa et al., 1987) as well as alternative polyadenylation (Northemann et al., 1989).

IL-6 is a pleiotropic HGF with biological effects on B cells (induces differentiation and antibody production) and T cells (induces activation, growth and differentiation). Maybe the most important physiological function of IL-6 is the regulation of the acute phase response. IL-6 plays another important role in hemopoiesis (the variety of biological activities is reviewed by Brakenhoff, 1990). Studies with recombinant IL-6 revealed that this HGF synergizes with IL-3 and GM-CSF in supporting colony formation of murine as well as human multipotential hemopoietic progenitors (Ikebuchi et al., 1987; Leary et al., 1988; Koike et al., 1988; Wong and Clark, 1988; Kishimoto, 1989). Although IL-6 affects early hemopoietic progenitor cells, by itself this factor does not

support colony growth. Thus, IL-6 is a potentiating factor by triggering early hemopoietic progenitor cells to become responsive to other HGFs. It has been suggested that IL-6 activates hemopoietic stem cells at the G_0 stage to enter the G_1 phase (Kishimoto, 1989).

IL-6 exerts its pleiotropic activities through binding to a high affinity IL-6 receptor (IL-6R) complex. The IL-6R complex consists of two membrane glycoproteins, i.e., an 80 kDa IL-6-binding protein and a 130 kDa (gp130) signal-transducing protein (Yamasaki et al., 1988; Saito et al., 1992). Molecular cloning data showed that both components of the IL-6R (the 80 kDa membrane protein and gp130) contain an immunoglobulin-like domain as well as a cytokine receptor superfamily domain. Similar to the G-CSFR, the IL-6R possesses characteristics of the immunoglobulin superfamily as well as of the hematopoietin or cytokine receptor superfamily (Bazan, 1990). The IL-6R does not contain sequences that show homology with any intrinsic tyrosine kinase domains. It has been shown that upon IL-6 stimulation, members of the JAK family of tyrosine kinases (JAK1 and JAK2) are activated which in turn translocate members of the STAT transcription factor family (STAT3) into the nucleus (Ihle and Kerr, 1995). It was shown that the JAK/STAT pathway of signal transduction is a common signaling pathway among cytokine receptors such as the receptors for IL-1 to IL-6, G-CSF, GM-CSF, Epo, IFNs, and many other growth factor receptors (reviewed by Ihle et al., 1994). In addition to membrane bound IL-6 receptors, soluble forms of the IL-6R have been identified (Tamura et al., 1993). Naturally occurring soluble forms of receptors have been identified for a variety of cytokine receptors and evidence is accumulating that they can act *in vivo* to regulate the biological activity of their ligand cytokines (Heaney and Golde, 1993).

Interleukin-7 (IL-7)

IL-7, also referred to as lymphopoietin 1 (LP-1) or pre-B cell proliferation factor, is a stimulator of B cell progenitors. A murine IL-7 cDNA from stromal cells was cloned by functional expression using the mammalian COS cell system (Namen et al., 1988). A cDNA encoding biologically active human IL-7 was isolated by hybridization with the homologous murine clone (Goodwin et al., 1989). Human IL-7 is a protein of 177 aa with six conserved cysteine residues and three potential N-glycosylation sites. The IL-7 protein was shown to be species specific. The genomic sequences were assigned to human chromosome 8 at band 8q12-13 (Sutherland et al.,1989b). As yet, the exact range of cell types responsive to IL-7 has to be elucidated, although studies with recombinant murine IL-7 clearly demonstrate that this factor plays a key role in the intramarrow differentiation of B cells. It has been proposed that stromal cell-dependent B cell development is subdivided into three stages: the first pro-B stage, which is independent on IL-7, the second early pre-B stage, which requires both IL-7 and other stromal signal(s) yet to be identified, and the third

mature pre-B stage which can proliferate in response to IL-7 alone (Sudo et al., 1989). The target cells of IL-7 are certainly not limited to the B cell lineage alone since also fetal thymocytes, pro-T-lymphocytes and mature T cells are influenced by IL-7 (Takeda et al., 1989; Morrissey et al., 1989). Thus, IL-7 is a pleiotropic HGF with a variety of activities affecting early events in lymphopoiesis (reviewed Henney, 1989).

The high affinity IL-7 receptor (IL-7R) is now known to consist of a specific IL-7 ligand-binding receptor (Goodwin et al., 1990) and the IL-2Rγ, a subunit of the trimeric IL-2 receptor complex. Apart from the IL-2R and IL-7R, this γ subunit is shared by the receptors of IL-4, IL-9, IL-13 and IL-15. The IL-7R belongs to the family of hematopoietin or cytokine receptor superfamily. In addition to the membrane-bound form of the IL-7 receptor, soluble forms capable of efficient binding of the IL-7 ligand have been identified (Goodwin et al., 1990). As alluded to earlier, naturally occurring soluble forms of receptors have been identified for a variety of cytokine receptors including the receptor for IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, G-CSF and GM-CSF (Heaney and Golde, 1993).

Interleukin-8 (IL-8)

IL-8 is a novel polypeptide that is produced by LPS-activated peripheral blood monocytes. It supports induction of neutrophil accumulation at the sites of inflammation. This neutrophil chemotactic and activating factor has variously been referred to as neutrophil attractant/activating protein (NAP-1), monocyte-derived neutrophil-activating factor (MONAP), monocyte-derived neutrophil chemotactic factor (MDNCF), neutrophil activating factor (NAF), T lymphocyte chemotactic factor (TCF), and leukocyte adhesion inhibitor (LAI). IL-8 belongs to a family of pro-inflammatory molecules called chemokines. These chemoattractants play a major role in mobilizing cellular defenses to fight off pathogens (Horuk, 1994). A cDNA encoding monocyte-derived neutrophil chemotactic factor (MDNCF/IL-8) has been identified (Matsushima et al., 1988) and used as a probe to isolate the complete genomic IL-8 sequences (Mukaida et al., 1989). Characterization of the complete nucleotide sequence of the IL-8 gene Mukaida et al., 1989) revealed that the 5' flanking region contains potential binding sites for several DNA binding proteins, including activation factor-1 and 2, IFN regulatory factor-1 and hepatocyte nuclear factor-1. The presence of these regulatory elements suggests that the expression of IL-8 is probably regulated by transcription factors interacting with the 5' flanking region of the IL-8 gene. However, the 5' flanking region of the IL-8 gene does not show sequences characteristic for 5' flanking region of genes encoding hemopoietic cytokines such as IL-2 to IL-5, GM- and G-CSF, TNFα and TNFβ. Furthermore, IL-8 lacks the well-conserved decanucleotide sequence "GPuGPuTTPyCAPy" present in the 5' flanking region of most hemopoietic growth factors. This observation, together with a more recent observation that

IL-8 is not capable to support colony formation of any type of hemopoietic cells (Mukaida et al 1989), suggests that IL-8 is not a member of the family of hemopoietic growth factors.

IL-8 exerts its biological activities by binding to specific cell surface receptors (reviewed by Horuk, 1994). Three distinct IL-8 receptors have been identified and were designated IL-8RA (type 1), IL-8RB (type B), and the red blood cell IL-8 receptor (RBC chemokine type). IL-8RA and IL-8RB are specific receptors that both bind IL-8 with high affinity. These receptors show approximately 77% aa sequence identity and the genes encoding these receptors are located in close proximity at chromosome 2q35. The RBC chemokine receptor is multispecific with respect to its ligands and binds, apart from IL-8, a variety of other chemokines as well. The precise function of these IL-8 receptors is not clear but it has been speculated that these different types of receptors activate differential signaling pathways (Horuk, 1994).

Interleukin-9 (IL-9)

IL-9 was identified by functional expression cloning in mammalian cells of a cDNA encoding a novel human hemopoietic growth factor (Yang et al., 1989) that appeared to be the human homolog of murine T-cell growth factor P40 (Van Snick et al., 1989). P40 was shown to be identical to a novel mast cell enhancing activity (MEA) isolated from the conditioned medium of a mouse T helper cell line. IL-9 is expressed by PHA/PMA stimulated C5MJ2 cells (Leary and Ogawa, 1987) and is capable of supporting proliferation of MO7E cells (Avanzi et al., 1988). The MO7E cells are, apart from IL-9, highly responsive to GM-CSF and IL-3 as well. Furthermore, IL-9 is produced by human T cell leukemia virus type I (HTLV-I) transfected T cell lines (C5MJ2 and C10MJ2). HTLV-I transformed T cell lines were shown to produce various lymphokines including GM-CSF, IFN-γ, IL-1α, IL-5 and IL-6. The HTLV-I genome contains a "pX" sequence in addition to the gag, pol and env genes required for virus replication. One of the proteins encoded by the "pX" sequence, designated as "p40x", was shown to activate the promoter in the HTLV-I LTR as well as many lymphokine promoters including those of GM-CSF, IL-2 and IL-3 transfected into T cells or non-T cells through a common transcription mechanism (Yokota et al., 1988). The human IL-9 cDNA encodes a precursor protein of 144 aa (Quesniaux, 1992). The cysteine rich mature protein (126 aa) contains four potential N-linked glycosylation sites and share 56% sequence homology with murine IL-9. Murine IL-9 was shown to be active on human cells, whereas human IL-9 does not seem to act on murine cells. The IL-9 gene has been mapped to band q31.2-q31.3 of human chromosome 5. This locus containing the genes encoding IL-3, IL-4, IL-5, IL-13, and GM-CSF is frequently deleted in patients with myelodysplasia or acute nonlymphoblastic leukemia (Le Beau et al., 1987).

IL-9 is a pleiotropic cytokine with multiple effects on cells of the lymphoid,

myeloid, erythroid, and mast cell lineages (reviewed by Quesniaux, 1992). IL-9 was shown to promote the growth of IL-3 responsive mucosal type mast cell lines. The *in vitro* survival of T cell lines is enhanced by IL-9. The IL-4 mediated stimulation of IgG, IgE and IgM production by normal B cells is potentiated by IL-9. Early erythroid progenitors (BFU-E) are stimulated by IL-9 in the presence of Epo (Donahue et al., 1990). IL-9 has been shown to be a potent inducer of proliferation for mouse thymic lymphomas *in vitro*. Recently, it was shown that IL-9 is a major anti-apoptotic factor for thymic lymphomas (Renauld et al., 1995).

High affinity IL-9 receptors were demonstrated on B cells, T cells, neutrophils and megakaryocytes. A cDNA encoding the IL-9 receptor (IL-9R) has been identified recently (Renauld et al., 1993). The extracellular domain of the IL-9R exhibits significant homology to other receptors of the hematopoietin or cytokine receptor superfamily. In addition, the IL-9R contains the WSXWS motif and four conserved cysteine residues which are characteristic for this cytokine receptor superfamily. The high affinity IL-9 receptor complex consists of two subunits, i.e., the IL-9R subunit and the IL-2Rγ subunit. Apart from the heterodimeric IL-9 receptor and the multimeric IL-2 receptor, the γ subunit is a common component of the receptor complexes for IL-4, IL-7, IL-13 and IL-15 (Russel et al., 1994).

Interleukin-10 (IL-10)

IL-10 was discovered on the basis of its ability to inhibit cytokine production by activated T cells. Initially, this factor was designated cytokine synthesis inhibitory factor (CSIF). Both murine and human IL-10 cDNAs have been identified (Vieira et al., 1991). Human IL-10 cDNA encodes a 178 aa precursor polypeptide with a signal peptide that is cleaved to generate a 160 aa mature protein with a predicted molecular weight of 18 kDa. Native human IL-10 is non-glycosylated and exists as a non-covalently-linked homodimer in solution (Quesniaux, 1992). The IL-10 gene has been mapped to chromosome 1 in mice as well as in humans. Although human IL-10 is active on mouse cells, mouse IL-10 has shown to be inactive on human cells.

IL-10 is a pleiotropic factor with immunostimulatory as well as immunosuppressive activities on a variety of cell types. As noted earlier, IL-10 inhibits the synthesis of cytokines by T helper cells in response to antigens. Furthermore, IL-10 inhibits synthesis of IFNγ and GM-CSF by PBMNC. Synthesis of IL-1, IL-6, IL-8 and TNFα by monocytes was shown to inhibited in the presence of IL-10. Stimulatory activities of IL-10 were demonstrated on thymocytes in the presence of IL-2 and IL-4. IL-10 stimulates mast cell growth in combination with IL-4 or IL-3 plus IL-4. The major biological effects of IL-10 on different cell types have been reviewed recently (Quesniaux, 1992).

A receptor for IL-10 has been isolated and cDNA analysis showed that it is a transmembrane glycoprotein of 100 kDa (Liu et al., 1994). The predicted aa

sequence of human IL-10R is 60% identical to mouse IL-10R. The human receptor binds human IL-10 with high affinity. Mouse IL-10 does not bind to this human IL-10R, an observation consistent with the known species specificity of IL-10. Both murine and human IL-10R are structurally related to the interferon-γ receptor (IFNR). Because IL-1- inhibits macrophage activation by IFN-γ, the resemblance between these receptors suggests that IL-10R may share a common receptor subunit with IFNR. Alternatively, the IL-10 and IFN signal transduction pathways may share common features. As yet, another subunit of the IL-10 receptor has not been identified. Moreover, the single chain IL-10R (Liu et al., 1994) binds IL-10 with high affinity, mediates transduction of a biological response to IL-10, and exhibits the known species-specificity of the human receptor. These observations indicate that the single chain IL-10R is a functional receptor for IL-10 (Liu et al., 1994)

Interleukin-11 (IL-11)

IL-11 is a pleiotropic cytokine that may play important roles in hemopoiesis, acute phase responses and in the development of adipocytes, neurons and osteoclasts (Du and Williams, 1994). The human IL-11 cDNA was isolated from a human fetal lung fibroblast cell line (Yang et al., 1991). The human IL-11 cDNA encodes a 199 aa precursor polypeptide with a 21 aa signal peptide. The mature IL-11 protein, generated by proteolytical processing of the precursor polypeptide, does not contain cysteine residues or potential glycosylation sites (Yang et al., 1991). Utilization of alternative polyadenylation sites results in two distinct transcripts of 1.5 and 2.5 kb. The gene encoding human IL-11 maps to the long arm of chromosome 19 at band 19q13.3-13.4 (Du and Williams, 1994).

IL-11 has multiple effects on both hemopoietic and nonhemopoietic cell populations (reviewed by Quesniaux, 1992; Du and Williams, 1994). In the presence of IL-3 or SCF, IL-11 supports erythropoiesis. IL-11 stimulates T cell dependent DNA synthesis and IgG secretion by B cells. As a single agent, IL-11 has no effects on murine megakaryocyte colony formation. However, IL-11 synergizes with IL-3 to enhance the number, size and growth of megakaryocyte colonies indicating that IL-11 stimulates multiple stages of megakaryocytopoiesis and thrombopoiesis. IL-11 was shown to act synergistically with IL-3, SCF or the combination of IL-3 and G-CSF to expand primitive long term culture-initiating cell (LTC-IC) populations. It has been suggested that the effects of IL-11 on early progenitor cells may be caused by triggering G_0 stem cells into cycle. IL-11, in combination with Epo and SCF, stimulates CFU-E. Furthermore, IL-11 synergizes with IL-3 to stimulate BFU-E growth. Thus, IL-11 may act directly on primitive erythroid progenitors and may promote their terminal differentiation. IL-11 induces the production of acute-phase reactants. IL-11 has significant effects, either direct or indirect, on nonhemopoietic cells, including neurons, small intestine crypt progenitor/stem cells, and pre-adipocytes (Du and Williams, 1994). Together, it is clear that IL-

11 has multiple effects on a large variety of cell types.

IL-11 exerts its biological activities through binding to specific high-affinity cell surface receptors. Recently, the ligand-binding subunit of the murine IL-11 receptor complex (IL-11Rα) has been cloned and characterized (Hilton et al., 1994). IL-11Rα is a low affinity receptor for IL-11 and belongs to the hematopoietin or cytokine receptor superfamily. It was shown that IL-11Rα interacts with gp130, which is a subunit of the high IL-6R complex, to generate the high affinity IL-11 receptor complex that is capable of transducing the IL-11 signal. Upon IL-11 stimulation, the JAK/STAT pathway of signal transduction is activated. JAK1 and JAK2 as well as STAT3 were shown to be involved in IL-11 signaling (Hilton et al.,1994).

Interleukin-12 (IL-12)

IL-12, also called natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF), was purified and subsequently cloned from human B-lymphoblastoid cell lines (Wolf et al., 1991; Gubler et al., 1991). IL-12 is structurally unique among the interleukins in that it is a heterodimer comprised of a 40 kDa (p40) and a 35 kDa (p35) subunit that are linked by a disulfide bond (Gubler et al., 1991). The human p40 protein is composed of 306 aa with 10 cysteine residues and 4 potential N-linked glycosylation sites. Native p40 contains approximately 10% carbohydrate. Human p35 comprises 197 aa with 7 cysteine residues and 3 potential N-linked glycosylation sites. Human p40 and p35 show, respectively 70% and 60% homology with their murine counterparts. The p40 and p35 subunits of IL-12 do not show significant sequence homology (Brunda, 1994). However, the p35 subunit has homology to IL-6 and G-CSF. In contrast, p40 does not show significant homology to any known cytokine but shows recognizable similarity to the extracellular domain of the IL-6R and the ciliary neurotrophic receptor (Brunda, 1994). Heterodimeric IL-12 is primarily produced by monocytes/macrophages which appear to constitutively express IL-12 mRNA. B cells and connective tissue type mast cells also produce IL-12 although less profoundly. The genes encoding the human IL-12 subunits are located on different chromosomes (Brunda, 1994) with the gene for p40 on chromosome 5 (5q31-q33) and the gene for p35 on chromosome 3 (3q12-q13).

IL-12 has multiple effects on both natural killer (NK) cells and T cells (reviewed by Quesniaux, 1992; Brunda, 1994). With respect to NK cells, IL-12 stimulates NK cell proliferation and upregulates the expression of membrane antigens. Furthermore, IL-12 is known to induce the production of IFN-γ and TNF-α in NK cells.The generation of LAK cells from NK cells depends on the presence of IL-12. Another interesting biological property of IL-12 is that it has potent antimetastatic and antitumor activities against a variety of experimental murine malignancies, probably mediated through immune mechanisms (Brunda, 1994). Recently, it was shown that IL-12 is a potent synergistic factor for

primitive hemopoietic stem cells (Jacobsen et al., 1993).

These distinct biological activities of IL-12 are mediated through binding of the heterodimeric IL-12 ligand to specific cell surface receptors. A cDNA encoding an IL-12 specific receptor (IL-12R) has been identified (Chua et al., 1994). Although human IL-12 p40 homodimer binds to this newly identified IL-12R, it does not mediate biological activity. Therefore, it has been suggested that this IL-12R is only a subunit of the functional IL-12R complex and that IL-12 signaling requires the binding of the IL-12 heterodimer (p40 linked to p35). This newly identified component of the functional IL-12 receptor complex shows homology (30%) with gp130 which is a subunit of the IL-6R complex (Chua et al., 1994).

Interleukin-13 (IL-13)

IL-13 is an interleukin-4-like cytokine that acts on monocytes and B cells, but not on T cells (Zurawski and De Vries, 1994). IL-13, originally called P600, has approximately 30% aa sequence homology with IL-4 as deduced from the recently identified cDNA encoding IL-13 (McKenzie et al., 1993). The human IL-13 cDNA encodes a 132 aa protein that has 66% nt sequence homology with murine IL-13 and the corresponding human and murine proteins show 58% aa sequence identity with five conserved cysteine residues. Analysis of cDNAs isolated from different sources demonstrated that polymorphic isoforms of IL-13 do exist (Zurawski and De Vries, 1994). A single 1.3 kb mRNA was exclusively detected in activated T cells. Despite multiple potential N-linked glycosylation sites, analysis of native IL-13 indicates that the protein is predominantly unglycosylated with a molecular weight of approximately 10 kDa. The human IL-13 gene maps to chromosome 5q31. This region at the long arm of chromosome 5 contains a cluster of HGFs that include GM-CSF, IL-3, IL-4, IL-5, and IL-9.

As alluded to earlier, IL-13 has IL-4-like activities such as profound effects on monocytes, surface antigen expression, antibody-dependent cellular cytotoxic activity, and cytokine synthesis. In addition, IL-13 has IL-4-like effects on human B cells (reviewed by Zurawski and De Vries, 1994). Unlike IL-4, IL-13 has no effect on either human or mouse T cells. Based on the observed biological activities, it may be concluded that IL-13, like IL-4 and IL-10, is a major anti-inflammatory cytokine that can suppress cell-mediated immunity. Furthermore, analysis of the biological effects of IL-13 showed that human and murine IL-13 are equally active on human cells, but human IL-13 has approximately 100-fold lower specific activity on mouse cells as compared to human cells. Determination of the actual role of IL-13 in immunological and inflammatory processes requires more information about the biological effects of IL-13 and about the signal transduction pathway of IL-13.

Similar to most HGFs, IL-13 exerts its biological effects through binding to specific receptors. As yet, a receptor for IL-13 has not been cloned. MAbs

specific for the 130 kDa chain of the high affinity IL-4 receptor (IL-4R) inhibit the effects of IL-4 as well as IL-13, suggesting that the functional IL-13 receptor may share a subunit with the IL-4R. The IL-4R does not bind IL-13 indicating that the ligand-binding subunits of the IL-4 and IL-13 receptor complexes are indeed distinct. Molecular cloning of an IL-13 binding subunit and characterization of the IL-13 receptor complex should clarify whether the functional IL-13 receptor is a heterodimer consisting of IL-4R and an IL-13 binding subunit.

Interleukin-14 (IL-14)
IL-14 is a growth factor with biological effects on B cells. This factor was initially called high-molecular-weight B cell growth factor (HMW-BCGF). Recently, a cDNA encoding human HMW-BCGF was identified (Ambrus et al., 1993). This group proposed to call this factor interleukin-14. The aa sequence of IL-14 deduced from cDNA predicts a secreted protein of 53 kDa with three potential N-linked glycosylation sites. A 15 aa signal peptide is cleaved from the precursor polypeptide to generate a 483 aa mature protein. The predicted IL-14 aa sequence has no significant homology to any known cytokine. The protein produced from the cDNA induces B cell proliferation, inhibits immunoglobulin secretion, and was recognized by Mabs directed against HMW-BCGF, indicating that this IL-14 cDNA encodes HMW-BCGF. IL-14 is expressed in activated normal T cells. Among blood cells, only B cells were shown to express receptors for HMW-BCGF/IL14 (Ambrus et al., 1993). Further studies on this novel cytokine are needed to elucidate its physiological role and to asses whether it plays a role in hemopoiesis and, especially its putative role in B cell proliferation.

Interleukin-15 (IL-15)
IL-15 is a novel cytokine, alternatively called ILT, which activates human natural killer (NK) cells via components of the IL-2 receptor. A cDNA encoding this novel cytokine has been cloned and expressed (Grabstein et al., 1994). The IL-15 precursor polypeptide (162 aa) contains a 48 aa leader peptide which is proteolytically cleaved to generate the mature IL-15 protein which has a predicted molecular weight of 14 kDa. The highest IL-15 mRNA levels were detected in epithelial cell lines, monocytes, muscle and placenta. This novel T cell growth factor has no homology with any other cytokine. Although this cytokine has no sequence identity to IL-2, IL-15 shares many of the T cell stimulatory activities described for IL-2. The biological activities attributed to IL-15 include stimulation of activated T cells, NK cells and LAK cells (Grabstein et al., 1994). Furthermore, it has been shown that IL-15 can stimulate the induction of B cell proliferation and differentiation (Armitage et al., 1995). Although the receptor for IL-15 has not been identified yet, many cell types including monocytes, T cells, B cells and endothelial cells bind IL-15

with high affinity. Analysis of IL-15 interaction with subunits of the IL-2 receptor revealed that the α subunit of the IL-2R is not involved in IL-15 binding. However, cells transfected with subunits of the IL-2R clearly demonstrated that both the β and γ chains are required for IL-15 binding and subsequent signaling (Giri et al., 1994). Hence, IL-15, like IL-2, IL-4 and IL-7 utilizes the common IL-2R γ chain. IL-15 and IL-2 are the only known cytokine that share the β signaling subunit of the IL-2R. Differential binding and response of some cell types to IL-2 and IL-15, suggests the existence of an additional, as yet unidentified, IL-15 specific receptor subunit.

1.3.3 Other cytokines involved in the modulation of blood cell production

In addition to the CSFs and ILs described in the previous section and summarized in Table 1.1, many other cytokines have been identified that were shown to be involved in the regulation of hemopoiesis.

Although interferons (IFNs) are primarily involved in interference of viral replication of infected cells, a variety of immunomodulatory activities mediated by IFNs have been documented. Three different types of IFN have been characterized: IFN-α, IFN-β and IFN-γ. IFN-α and IFN-β are structurally related 20 kDa proteins that bind to the same receptor, whereas, IFN-γ is structurally distinct as reflected by its binding to a separate receptor (Aguet et al., 1988). Most of the activities attributed to IFNs are believed to be mediated by IFNs induced proteins. With regard to hemopoiesis, IFN-γ was shown to strongly inhibit the proliferation and differentiation of hemopoietic bone marrow progenitors *in vitro* (Fisher et al., 1986; Hosoi et al., 1985). IFNs exert an antiproliferative effect on some factor dependent myeloid cell lines by inhibition of the expression of cell proliferation-associated genes such as the nuclear proto-oncogene c-*myc*, which was shown to be inhibited by IFN-γ, either by destabilization of the mature mRNA (Dani et al., 1985), or by impaired post-transcriptional splicing processes (Harel-Bellan et al., 1988). Thus, IFNs seem to function as negative regulators of the proliferation and differentiation of hemopoietic cells.

Tumor necrosis factor-α (TNF-α), initially referred to as cachectin, and TNF-β (lymphotoxin) are structurally and functionally related 17 kDa proteins primarily involved in inflammatory reactions. The genes encoding these proteins are both located within the major histocompatibility complex on the short arm of the human chromosome 6 (Steel and Hutchins, 1989). The two TNF proteins have the ability to induce or suppress the expression of a vast number of genes, including those for HGFs and their receptors, transcription factors, inflammatory modulators and acute phase proteins. Both TNF proteins may support or inhibit bone marrow colony formation *in vitro* (Akahane et al.,

1987). In addition, TNF-α was shown to preferentially suppress erythropoiesis (Johnson et al., 1989; Johnson et al., 1990). However, it is not clear whether the TNF proteins directly affect hemopoiesis or that the observed effects have to be attributed to TNF induced release of HGFs such as GM-CSF, M-CSF and IL-1 (Kaushansky et al., 1988).

Transforming growth factor-β (TGF-β) proteins (TGF-β1 to TGF-β5) are members of a family of closely related polypeptides of 12.5 kDa, usually occurring as disulfide-linked dimers (Steel and Hutchins, 1989). It has been demonstrated that TGF-β acts *in vitro* directly on primitive hemopoietic progenitors resulting in inhibition of colony formation, while more committed progenitors were not affected (Keller et al., 1990). It has been hypothesized that TGF-β plays a dual role in hemopoiesis by inhibiting proliferation of immature precursors and by promoting differentiation of committed precursors, especially of the monocytic lineage (Keller et al., 1990).

Leukemia inhibitory factor (LIF) is identical to a factor that supports the proliferation of murine interleukin-3 dependent leukemic DA-1a cells (Moreau et al., 1988). Therefore, this novel factor has also been termed human interleukin for DA cells (HILDA) . The cDNAs encoding murine (Gearing et al., 1987) as well as human LIF (Gough et al., 1988) have been identified and expressed and the genes have been assigned to murine chromosome 11 (Kola et al., 1990) and human chromosome 22q12 (Sutherland et al., 1989), respectively. LIF, a glycosylated polypeptide of 20-40 kDa, was initially identified as a cytokine defined by its capacity to induce macrophage differentiation of certain myeloid leukemic cells (Gearing et al.,1987; Gough et al., 1988). In addition, it has been demonstrated that LIF can affect normal hemopoiesis as well. Administration of recombinant murine LIF to mice resulted in elevated number of megakaryocyte progenitors in the spleen, followed by an increase in the numbers of spleen as well as of bone marrow megakaryocytes, and finally a rise in platelet levels, suggesting a role in megakaryocytopoiesis and thrombopoiesis (Metcalf et al., 1990). The high affinity receptor for LIF consists of a low affinity LIF binding subunit and the common gp130 signal-transducing subunit which has shown to be involved in signal transduction of IL-6, IL-11, ciliary neurotrophic factor (CNTF), and oncostatin M (OSM) as well (Ihle and Kerr, 1995). LIF inhibits differentiation and stimulates proliferation of multipotential embryonic stem (ES) cells while maintaining their totipotency (Williams et al., 1988). The molecular cloning and large-scale production of LIF allowed *in vitro* cultures of ES cells which facilitated gene targeting experiments to study the effects of "knocking out" genes in mice (Joyner et al ., 1989).

Another group of inducible inflammatory molecules, called chemokines, are involved in a variety of immune and inflammatory responses. Members of this superfamily of relatively small (8-10 kDa) pro-inflammatory cytokines act primarily as chemoattractants and activators of specific leukocytes. The general function appears to be recruitment and activation of leukocytes at the site of

inflammation. Apart from IL-8, identified as a potent neutrophil chemoattractant (Horuk, 1994), macrophage inflammatory protein-1α (MIP-1α) has been identified as an important member of this family of chemokines. MIP-1α, a potent chemoattractant of monocytes, appeared to be identical to a hemopoietic stem cell inhibitor (SCI) that was shown to inhibit the proliferation of hemopoietic stem cells both *in vitro* and *in vivo* (Graham et al., 1990). In contrast, SCI/MIP-1α does not affect the proliferation of less primitive progenitors such as GM-CFU and therefore appears to be a specific regulator of the stem cell compartment. SCI/MIP-1α has been classified as a hemopoietic "negative regulator", that is responsible for the quiescent state with respect to DNA synthesis of stem cells and early progenitor cells (Axelrad, 1990).

A variety of receptor-type tyrosine kinases are expressed by hemopoietic cells. Among them, the proto-oncogenes c-fms, c-kit and c-mpl were shown to be the ligand binding receptors for M-CSF, SCF and thrombopoietin, respectively (see previous sections; 1.3.1). These ligands play an important role in the regulation of hemopoiesis. Studies conducted to identify stem cell specific genes encoding tyrosine kinase-type receptors resulted in the molecular cloning of a new tyrosine kinase receptor designated flt3 or alternatively called flk-2 (Rosnet et al., 1991; Matthews et al., 1991). This flt3/flk-2 receptor was found to be expressed on primitive hemopoietic progenitors and lymphoid precursors suggesting that the flt3 ligand (FL) may play an important role in early lymphopoiesis. Indeed, recombinant murine FL in combination with IL-6, SCF, IL-11, or G-CSF supported the proliferation of primitive lymphohemopoietic progenitors. FL, as a single agent and in synergy with SCF or IL-7, supported the proliferation of B cell progenitors (Hirayama et al., 1995). Recently, the human homologue of murine FL has been cloned (Lyman et al., 1994). The human protein shows 72% aa identity to murine FL and was shown to be cross-reactive. Human FL stimulates the proliferation and supports colony formation of human bone marrow cells enriched for hemopoietic progenitors and that are CD34+. The exact array of biological activities exerted by FL has to be determined yet.

1.4 Interleukin-3

1.4.1 Biochemical and genetic features of interleukin-3

Interleukin-3 (IL-3) was initially purified to homogeneity from medium conditioned by myelomonocytic leukemia WEHI-3 mouse cells (Ihle et al., 1982; Ihle et al., 1983). Characterization of the homogeneous protein showed that murine IL-3 (mIL-3) is a protein which migrates as a broad band (20 to 30 kDa) upon SDS polyacrylamide gel electrophoresis. The native protein is glycosylated (approximately 38% carbohydrate) and the major glycosylated molecule has an apparent molecular weight of 28 kDa with an isolelectric point (pI) of 4-8 (Ihle, 1989). Furthermore, native mIL-3 appeared to be relatively

stable under a wide variety of conditions including pH and temperature. Protein sequencing of native mIL-3 indicated that the secreted protein, either starts at the proposed first aa (Ala at pos. 27; Clark-Lewis et al., 1984) or starts 6 residues further downstream (Asp at pos. 33; Ihle et al., 1983), the latter protein is most likely the result of proteolytic cleavage. The isolation and expression of a cDNA encoding murine IL-3 (Fung et al., 1984; Yokota et al., 1984; Kindler et al., 1986) facilitated the large-scale production of murine IL-3 (mIL-3) glycoprotein required for its biochemical characterization. The cDNA sequence shows a single open reading frame that specifies a polypeptide of 166 amino acids (aa). This precursor protein was shown to be processed at the N-terminal site by the removal of 26 residues to yield a mature protein comprising 140 aa. The protein sequence of mature processed mIL-3 indicated a molecular weight of 15,102 dalton with four potential N-linked glycosylation sites and four cysteine residues. Initial experiments indicated that glycosylation is not required for biological activity (Kindler et al., 1986). Biological active mIL-3 has been chemically synthesized (Clark-Lewis et al.,1986), further demonstrating that the carbohydrate moieties are not essential for its activity *in vitro*. However, it remains to be elucidated whether glycosylation affects the biological activity or stability *in vivo*. Characterization of the synthetic analogues established that the functional domain is localized in the amino terminal fragment and the cysteine residue at position 17 is essential for activity. Moreover, it was shown that a single disulfide bridge, between cysteines 17 and 80, is required for full biological activity (Clark-Lewis et al., 1988). Apparently, the favorable functional conformation is stabilized by this disulfide bond.

Isolation of the IL-3 gene from a mouse genomic library using a full length IL-3 cDNA revealed that this relatively small gene, comprising 2193 bp, is composed of four introns and five exons (Miyatake et al., 1985). In the 5'-flanking region of the gene, several structural elements were identified which have been shown to be important features of eukaryotic promoters. These features include a "TATA"-like sequence (Benoist and Chambon, 1981) preceded by a G+C-rich region (From and Berg, 1982; Treisman et al., 1983) and a "CAT"-related sequence. There are nine repeats of a closely related 14 bp sequence in the second intron of the murine IL-3 gene. This motif was identified at an identical place in the gene encoding rat IL-3 (Cohen et al., 1986) and shows homology with reported enhancer elements (Miyatake et al., 1985). Whether this repeated 14 bp consensus sequence observed in several other genes (Cohen et al., 1986) and associated with "hypervariable regions" of DNA, indeed functions as an enhancer remains to be investigated. The coding regions for mature rat and mouse IL-3 share 76% sequence homology, whereas the flanking regions and introns display 90% and 80% nucleotide homology, respectively, with the intron/exon junctions fully conserved. The low amino acid homology of 54% for mature rat and mouse IL-3 correlates with the observed

species specificity, i.e., mouse IL-3 fails to stimulate rat bone marrow progenitor cells and rat IL-3 fails to stimulate mouse bone marrow progenitor cells (Cohen et al., 1986). The low sequence homology and lack of interspecies cross-reactivity suggests that IL-3 has evolved rapidly.

The single-copy IL-3 gene (Miyatake et al., 1985; Cohen et al., 1986) as well as the GM-CSF gene (Stanley et al., 1985) have been assigned to mouse chromosome 11 (Barlow et al., 1987; Ihle et al., 1987). Fine-structure mapping by pulsed-field gel electrophoresis of the region around the IL-3 gene shows that the IL-3 and GM-CSF genes are closely linked. The IL-3 gene is located in the same orientation only 14 kb upstream to the GM-CSF gene (Lee and Young, 1989). The close linkage of the IL-3 and GM-CSF genes together with structural and functional overlaps suggest that these genes have evolved from a common ancestral gene or that the two genes have somehow been juxtaposed to coordinate their expression (Lee and Young, 1989). It is noteworthy that IL-4 and IL-5 also map to mouse chromosome 11 at the same band position (Lee and Young, 1989). Notably, a variety of HGF genes, including GM-CSF, IL-3, IL-4, IL-5, IL-9, the p40 subunit of IL-12 and IL-13 are clustered on the long arm of the human chromosome 5 which is the human counterpart of murine chromosome 11 (see Table 1.2, section 1.3.1 to 1.3.2, and chapters 3, 4 and 6). The significance of the clustering of functional closely related genes and the question as to whether this chromosomal association permits a possible coordinated expression of these genes is as yet unclear.

Among the known HGFs, IL-3 is the only factor to be predominantly if not exclusively produced *in vitro* by activated normal T cells (Otsuka et al., 1988; Niemeyer et al., 1989). The same cell specificity exists for the production of both human and murine IL-3. This exclusive association of IL-3 gene expression with activated T cells is in contrast with the variety of cellular sources for GM-CSF. In spite of expression of IL-3 and GM-CSF in T cells, endothelial cells and stromal fibroblasts produce GM-CSF in response to IL-1 or TNF while under these conditions IL-3 expression was not detected (Sieff et al., 1987a). The exclusive production of IL-3 by activated T cells has led to the hypothesis that IL-3 is not involved in normal hemopoietic differentiation but is associated with hemopoietic stem cell differentiation where its regulation is mediated by environmental response of the immune system (Ihle, 1989). Studies on IL-3 expression in human T cell clones revealed that IL-3 expression is restricted to a small subset of T cells and NK cells and that IL-3 expression can be enhanced by mitogens and antigens, or even more profoundly by IL-2 (Wimperis et al., 1989). An exception to the T cell restricted IL-3 production is the WEHI-3 myelomonocytic cell line where the constitutive production was shown to be due to the insertion of an endogenous retrovirus-like element with its 5' long terminal repeat (LTR) close to the promoter region of the IL-3 gene (Ymer et al., 1985). Recently, it has been shown that in addition to activated T cells other cell types such as activated murine mast cells, murine keratinocytes, eosinophils,

neutrophils, thymic epithelial cells, neurons and astrocytes are capable of IL-3 expression (Kita et al., 1991; Konishi et al., 1995). Although low expression levels of IL-3 could be detected in these cells, as yet activated T cells are the predominant natural source of IL-3.

The mechanisms involved in the regulation of IL-3 gene expression in T cells are largely unknown. It has been demonstrated that IL-3 mRNA expression in the CD28+ subset of normal human T cells is induced by activation through the T cell receptor (TCR)/CD3 complex but not through the alternative T cell activation pathway using phorbol myristate acetate (PMA) and anti-CD28. However, IL-3 expression could be induced by costimulation of T cells with both PMA and the calcium ionophor ionomycin. Based on these results, the investigators (Guba et al., 1989) conclude that the IL-3 gene induction pathway is obligatory calcium dependent. Regulation of IL-3 mRNA expression in mast cells seems to occur at the posttranscriptional level and is mediated by calcium ions (Wodnar-Filipowicz and Moroni, 1990).

Since the genes encoding GM-CSF and IL-3 are localized in close proximity on the same chromosome and as these factors are both produced by T cells following activation of the TCR complex, it has been suggested that these factors are expressed through a common mechanism (Barlow et al., 1987). Initially, a highly conserved decanucleotide sequence with the consensus sequence "GPuGPuTTPyCAPy" present in the 5' flanking region of the genes encoding GM-CSF and IL-3 has been postulated to account for the coordinate expression of these genes in activated T cells (Stanley et al., 1985). The presence of this well-conserved decanucleotide in other T cell expressed growth factor genes, including the genes coding for IFN-γ, IL-2 and the IL-2 receptor, supported the postulated role that this sequence mediates transcriptional activation of IL-3 and GM-CSF in T cells. However, the presence of this regulatory element in the genes for both human and murine G-CSF, a factor that is not produced by activated T cells, makes it unlikely that this sequence alone is responsible for T cell expression of IL-3 and GM-CSF. Another sequence with the consensus "TCAGPuTA" located at the 3' end of the decanucleotide was found to be conserved in both human and murine IL-3 and GM-CSF, but not in G-CSF or IL-2 (Shannon et al., 1988). The promoter regions of IL-3 and GM-CSF, spanning these two motifs were shown to bind a T cell specific nuclear protein. Studies on the precise role of these responsive elements as well as studies on the regulation of the expression or activity of these transcription factors may provide insights in the mechanism of regulation of the lymphokine genes.The regulation of IL-3 in primary human T lymphocytes, the identification of a number of regulatory elements in the 5' upstream region as well as in the 3' untranslated region of the human IL-3 gene and their conservation among other species and the role of positive and negative transcriptional factors involved in IL-3 regulation are discussed in chapters 3, 4, 6 and 7.

1.4.2 Biological properties of interleukin-3

Interleukin-3 (IL-3) was initially defined as a T cell derived factor which could induce the expression of the T cell associated enzyme 20-α-hydroxysteroid dehydrogenase (20-α-SDH) in populations of cells containing T cell precursors (Ihle et al., 1981). Besides the ability to induce 20-α-SDH, homogeneous murine IL-3 exerts a broad spectrum of biological properties (Ihle et al., 1983) including stimulatory activity on WEHI-3 cells as well as on several other myeloid leukemia cell lines, stimulatory activity on the formation of granulocyte-macrophage colonies, mast cell growth factor activity, P cell-stimulating activity, and histamine-producing cell-stimulating factor activity (Ihle et al., 1983). In addition, IL-3 is capable to promote the proliferation of megakaryocytic colony-forming cells (Ishibashi and Burstein, 1986), to support the differentiation of eosinophils (Sanderson et al., 1985), to support the growth of pre-B cell precursors (Palacios et al., 1984), to promote the proliferation of natural cytotoxic (NC) cells but not natural killer (NK) cells (Djeu et al., 1983) and to promote the formation of osteoclasts (Scheven et al., 1986). Murine IL-3 was shown to stimulate stem cell self-renewal and therefore called stem cell activating factor (Dorssers et al., 1984). Thus, IL-3 acts on a wide variety of hemopoietic cells including pluripotential hemopoietic stem cells and multiple types of hemopoietic progenitor cells (Suda et al., 1985; Spivak et al., 1985; Hapel and Young, 1988). There seems to be no potential role for IL-3 in the regulation of lymphoid lineage differentiation. It is clear that IL-3 does not potentiate the proliferation of committed T or B cells or of prothymocytes (Mulder et al., 1985).

The molecular cloning of murine IL-3 and its subsequent large-scale production enabled much more refined studies *in vitro* as well as *in vivo* with this recombinant HGF to elucidate its physiological role. Recombinant murine IL-3, produced by bacteria, exerted biological *in vitro* effects identical to those observed with homogenous native murine IL-3 in terms of target cell specificity, spectrum of effects and specific activity. Thus, the carbohydrate component of the mature molecule is not required for its biological activity. Recombinant bacterial murine IL-3 enhanced hemopoiesis after administration of IL-3 to normal (2 fold) as well as sublethally irradiated mice (>10 fold). The latter indicates that decreased hemopoietic activity resulting from irradiation can apparently be compensated for by exogenous IL-3, which is an especially interesting observation in terms of treatment of chemotherapy and radiation induced pancytopenia. Furthermore, these *in vivo* studies demonstrated that erythropoiesis was as strongly enhanced as granulopoiesis. Studies on retrovirus-mediated transfer and expression of the interleukin-3 gene in mouse hemopoietic cells following transplantation in irradiated mice demonstrated a marked elevation in leucocyte counts, bone marrow hyperplasia and enlargement of the liver and spleen as a consequence of endogenous IL-3

production (Wong et al., 1989). These results are in concordance with those observed in analogous *in vivo* studies with exogenous IL-3 administration.

It has been shown that IL-3 acts synergistically with other HGFs including M-CSF, GM-CSF, G-CSF, SCF, Epo, IL-1, IL-2, IL-4, IL-5, IL-6, IL-9 and IL-12 to support the proliferation and differentiation of hemopoietic progenitors of various lineages. IL-3 synergizes with M-CSF to stimulate the *in vitro* growth of primitive murine and human bone marrow progenitors (Koike et al., 1986; Zhou et al., 1988; Morris et al., 1990). IL-3 acts synergistically with recombinant G-CSF and GM-CSF on multipotential human (Paquette et al., 1988) as well as murine (Ikebuchi et al., 1988) bone marrow progenitors to stimulate their proliferation. Erythropoietin acts synergistically with IL-3 on erythroid progenitors to support the development of hemoglobinised erythroid colonies (Barber et al., 1989). GM-CSF and, especially, IL-3 significantly enhanced the proliferation of IL-2 responsive cells (Santoli., 1988). Connective tissue type mast cells (CMTC) are dependent for their development on the presence of IL-3 and IL-4, while either factor alone did not stimulate CMTC proliferation (Tsuji et al., 1990). Thus IL-3 frequently acts in combination with other HGFs to exert specific biological activities.

The biological and physiological properties of human and rhesus monkey interleukin-3 will be presented and discussed in chapter 5. Furthermore, the biological activities of chimpanzee, tamarin and marmoset IL-3 are discussed in chapter 6. The cross-reactivity of IL-3 among species is discussed in chapter 5 and 6.

1.4.3 Interleukin-3 cell surface receptor

At the time murine (see section 1.3.1) and human IL-3 (see chapter 3) were identified, it was largely unknown how interleukin-3 exerts its multiple biological activities on responding cells. It has been demonstrated that the primary stimulus triggering a variety of signal-transduction events, is the binding of IL-3 to a specific high affinity cell surface receptor. Chemical cross-linking experiments between radiolabeled murine IL-3 and plasma membranes demonstrated the presence of IL-3 specific membrane glycoproteins of various molecular weights, including a 140 kDA membrane glycoprotein (Sorensen et al., 1986; May and Ihle, 1986; Nicola and Peterson, 1986; Isfort et al., 1988a,b; Sorensen et al., 1989). The molecular weight discrepancies reported initially were shown to be due to cleavage of the cross-linked complex by a plasma membrane-associated protease (Murthy et al., 1990). Based on the results of the chemical cross-linking experiments, it was proposed that he murine IL-3 receptor is 140 kDa membrane glycoprotein that was shown to be rapidly tyrosine phosphorylated upon IL-3 stimulation (Sorensen et al., 1989; Isfort et al., 1988a,b; Murthy et al., 1990).

Analysis of receptor-binding studies between mIL-3 and growth factor-

dependent cell lines demonstrated the presence of a single class of high-affinity cell surface receptors (dissociation constant (K_d) = 50-200 pM) with a relatively low abundance of 400-5000 receptors per cell (Park et al., 1986; Nicola and Metcalf, 1988). However, evidence for a low-affinity mIL-3 receptor (K_d = 18 nM) has been reported also (Schreurs et al., 1990). Following binding of mIL-3 to the receptor expressed on normal bone marrow cells and a number of different cell lines, rapid internalization of occupied receptors was seen (Nicola and Metcalf, 1988; Nicola et al., 1988). Upon internalization, mIL-3 was shown to be degraded in lysosomes while its receptor is recycled to the cell surface (Nicola et al., 1988; Murthy et al., 1989). Furthermore, it has been demonstrated that IL-3 has the capacity to down-regulate its own receptor (Nicola and Metcalf, 1988; Murthy et al., 1989).

Initially, a gene (AIC2A) encoding a low affinity mIL-3 receptor was isolated (Itoh et al., 1990). However, AIC2A did not appear to be functional in terms of ligand-dependent internalization or stimulation of tyrosine phosphorylation. Since a consensus sequence for a tyrosine kinase was not present in the cytoplasmic domain it was suggested that additional polypeptide subunits are required for a functional high-affinity receptor. Indeed, ligand affinity purification of a murine IL-3 binding protein complex showed that AIC2A, a 120 kDa glycoprotein, is a component of the murine high affinity IL-3 cell surface receptor(Schreurs et al., 1991). Another cDNA (AIC2B), encoding a protein that is 91% identical to AIC2A but that not binds murine IL-3, was identified (Gorman et al., 1990). A third cDNA was cloned that encodes a 70 kDa membrane glycoprotein (SUT-1) that binds mIL-3 with low affinity (Hara and Miyajima, 1992). Association of this subunit of the IL-3 receptor (referred to as the IL-3Rα subunit) with AIC2B, now referred to as the common β subunit (β_c) confers a high affinity murine IL-3 receptor. The β_c subunit is shared by the functional murine IL-5 and GM-CSF receptors (Kitamura et al., 1991; Miyajima et al., 1993). Also the AIC2A subunit that binds IL-3 with low affinity, now referred to as the IL-3 specific β subunit (β_{IL3}) is converted into a functional high affinity IL-3 receptor after association with the IL-3Rα subunit (Hara and Miyajima, 1992). However, AIC2A (β_{IL3}) is not shared by the murine receptors for IL-5 and GM-CSF. Although human homologs of SUT-1 and AIC2B have been identified (Kitamura et al., 1991), a human homolog of AIC2A has not been found yet. The nature of the existence of two functional high affinity murine IL-3 receptors is not clear. The AIC2 proteins and the SUT-1 protein belong to the hemopoietin or cytokine receptor superfamily that includes two subunits (β and γ) of the IL-2 receptor and receptors for IL-4, IL-5, IL-6, IL-7, IL-9, GM-CSF, G-CSF, Epo, LIF, Oncostatin M, and ciliary neurotrophic factor (Bazan, 1990; Miyajima et al., 1993; Ihle et al., 1994; Ihle and Kerr, 1995). Members of this hemopoietin/cytokine superfamily have a common structural motif of approximately 200 aa in their extracellular domains. Characteristic features

within this motif include the presence of four highly conserved cysteine residues and the presence of a unique sequence tryptophan-serine-x-tryptophan-serine (the WSXWS motif).

At the time that human IL-3 was identified (see chapter 3), little was known about the molecular structure of the human IL-3 cell surface receptor. The existence of both high and low-affinity receptors on a variety of human normal hemopoietic cells as well as on a number of human leukemic cell lines was demonstrated (Park et al., 1989a,b; Valent et al., 1989; Elliott et al., 1989; Kuwaki et al., 1989). Analysis of the cellular distribution of IL-3 receptors on normal human cells revealed low to moderate expression on monocytes and bone marrow cells (150 and 50 receptors per cell, respectively), whereas IL-3 receptors were not found on neutrophils (Park et al., 1989a; Lopez et al., 1988; Lopez et al., 1989). In addition, high affinity IL-3 binding sites were found on human blood basophils (Valent et al., 1989), indicating a potential role for IL-3 as a regulator of basophil function. The binding characteristics of IL-3 and GM-CSF to their receptors on various target cells provided evidence for the existence of distinct receptors for these HGFs with approximate molecular masses of 70 and 93 kDa, respectively (Gesner et al., 1989). On murine hemopoietic cells the binding of GM-CSF to its receptor is not directly competed for by IL-3 (Walker et al., 1985). However, in the human system it is well established that human GM-CSF binding is competed for either by IL-3 or IL-5, a phenomenon known as cross-competition (Lopez et al., 1989; Park et al., 1989b; Elliott et al., 1989; Budel et al., 1990; Lopez et al., 1991). This cross-competition suggested the existence of a shared receptor subunit for IL-3, IL-5 and GM-CSF. Indeed, it was shown that analogous to the murine IL-3 cell surface receptor, the functional human IL-3 receptor is a heterodimeric molecule consisting of two distinct subunits (Kitamura et al., 1991; Miyajima, 1992; Miyajima et al., 1993). Identification of the human IL-3 cell surface receptor subunits, characterization of the functional heterodimeric IL-3, IL-5 and GM-CSF receptors, and potential implications for the species specificity and the molecular evolution of IL-3 are presented and discussed in the next chapters.

1.4.4 Interleukin-3 signal transduction

It was shown that IL-3 exerts its pleiotropic activities through binding to a specific high affinity cell surface receptor (see previous section). Although the IL-3 receptor lacks kinase domains the receptor couples IL-3 binding with the induction of tyrosine and serine phosphorylation of specific cellular proteins (Isfort et al., 1988a,b; Morla et al., 1988; Koyasu et al., 1987; Sorensen et al., 1989). The rapid induction of tyrosine phosphorylation is a common observation following ligand binding of many HGFs including IL-3, IL-4, GM-CSF, G-CSF, M-CSF, IL-2, TNF-α and IFN-γ (Isfort and Ihle, 1990; Evans et al., 1990). These observation have led to the hypothesis that a tyrosine kinase

physically associates with the receptor upon ligand binding. In addition to tyrosine kinases, protein kinase C activation without increasing inositol lipid turnover is likely to be involved in the IL-3 mediated proliferation of hemopoietic cells (Whetton et al., 1988).It was shown that the β subunit of the IL-3R is involved in IL-3 signaling. In addition to a number of intracellular proteins, the β subunit itself is tyrosine phosphorylated upon ligand binding (Ihle and Kerr, 1995). The observation that IL-3 ligand binding results in tyrosine phosphorylation of SHC a protein involved in the activation of the $p21^{ras}$ proto-oncogene, indicated that the Ras signaling pathway is involved in IL-3 signaling (Satoh et al., 1991). The activation of $p21^{ras}$ include tyrosine phosphorylation of SHC, its association with an adaptor protein (Grb2) and with a G protein nucleotide exchange factor (SOS), an increase in ras-bound GTP, activation of raf-1, activation of the mitogen activated protein (MAP) kinase cascade, and induction of the expression of early response genes such as c-*myc*, c-*fos* and *jun*B (reviewed by Ihle and Kerr, 1995). The enhanced expression of early response genes is in concordance with previous observations that IL-3 induces the expression of these proto-oncogenes (Whetton et al., 1988; Cleveland et al., 1989; Ihle, 1990).

In addition to the Ras signal transduction pathway, a second distinct pathway, the JAK/STAT mechanism of signal transduction has been implicated with IL-3 signaling (Silvennoinen et al., 1993; Ihle et al., 1994; Ihle and Kerr, 1995; Azam et al., 1995). Recent studies have shown that the induction of tyrosine phosphorylation upon cytokine ligand binding is mediated by members of the Janus kinase (JAK) family of cytoplasmic protein tyrosine kinases. Although JAK was initially an acronym for just another kinase, it has also been proposed as an acronym for Janus kinase. The most striking feature of members of the JAK family is the presence of two kinase domains. The more C-terminal domain is believed to have protein tyrosine kinase catalytic activity. JAKs physically associate with the cytokine receptor and become activated following ligand binding. Autophosphorylation is associated with the activation of JAK kinase activity. The activated kinases then phosphorylate the receptor as well as a number of cellular substrates including cytoplasmic proteins belonging to a family of transcription factors called the signal transducers and activators of transcription (STATs).

Activation of the JAK/STAT signaling pathway by single chain receptors such as those for Epo, G-CSF, growth hormone and prolactin is initiated by dimerization and oligomerization of the cognate receptor. The current model for activation of JAKs by heterodimeric or heterotrimeric receptors such as those for IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-11, IL-12, CNTF, LIF, IFNs and GM-CSF envisions ligand binding to the specific α chain and its subsequent association with the common β subunit or the common gp130 signaling subunit. These ligand/receptor complexes further dimerize and oligomerize resulting in the association of JAKs with the β chain. Rather complex mechanisms of JAK

activation have been described: i) a single receptor chain associates with a single JAK; ii) alternatively a single receptor chain may associate with multiple JAKs; iii) individual receptor chains associate with specific JAKs (Ihle and Kerr, 1995).

An attractive working hypothesis of the JAK/STAT pathway of IL-3 signaling has been reported (Ihle et al., 1994; Ihle and Kerr, 1995). The proposed sequence of events that occurs following IL-3 binding is: I) IL-3 binding to its ligand specific IL-3Rα subunit is associated with increased affinity of the proximal region of the common β subunit (β_c) of the dimerized and oligomerized IL-3R complex for JAK2 (Silvennoinen et al., 1993); II) JAK2 is recruited to the IL-3R receptor and associates with β_c to become subsequently autophosphorylated; III) the phosphorylation of JAK2 is associated with the activation of JAK2 kinase activity; IV) the activated kinases then phosphorylate the IL-3 receptor; V) a number of cellular substrates, including STAT5 (Azam et al., 1995; Mui et al., 1995), are recruited to the receptor/kinase complex and are subsequently phosphorylated. Phosphorylation of STATs leads to the formation of STAT dimers which activate transcription. Thus phosphorylation-dependent dimerization is required for STAT DNA-binding and subsequent transcriptional activation of gene expression.

Apart from the Janus kinases and tyrosine kinases implicated in the Ras pathway of signal transduction, a number of other tyrosine kinases have been identified that are activated by IL-3. The proto-oncogene product c-fps/fes and lyn kinase were shown to be activated upon IL-3 or GM-CSF stimulation (Torigoe et al., 1992; Hanazono et al., 1993). In addition, it was shown that Tec protein tyrosine kinase is involved in IL-3 signaling (Mano et al., 1995). Tec is a nonreceptor type cytoplasmic protein-tyrosine kinase and does not belong to the Src family of tyrosine kinases. Stimulation with IL-3 induces rapid tyrosine phosphorylation of Tec in both myeloid and pro-B-cells. In addition IL-3 stimulation induces kinase activity of Tec itself. The role of these protein-tyrosine kinases in IL-3 signaling and whether all these kinases are involved in the Ras-induced MAP kinase cascade has to be elucidated. GATA-2, a transcription factor expressed in hemopoietic stem cells, was shown to be rapidly tyrosine phosphorylated upon IL-3 stimulation (Towatari et al., 1995). Notably, it was suggested that a MAP kinase may phosphorylate GATA-2. Activation of protein kinase C (PKC) has been observed following IL-3 stimulation (Rao et al., 1995). It was suggested that PKC is a downstream effector of the IL-3 mediated phosphorylation in the Ras signal transduction pathway.

1.5 Outline of this thesis

Murine interleukin-3 (mIL-3) was shown to be a potent stimulator of hemopoiesis *in vitro*. The broad spectrum of biological activities of this HGF

observed *in vitro* included the stimulation of the growth and differentiation of hemopoietic progenitors and multipotential stem cells. The molecular cloning and expression of the mIL-3 gene enabled large-scale production required for *in vivo* stimulation of hemopoiesis and facilitated a detailed *in vivo* analysis of its role in murine hemopoiesis. From these studies it was apparent that the retrieval of a human equivalent to recombinant mIL-3 would be an especially important goal to achieve since it could be expected that recombinant human IL-3 could be of considerable benefit for the treatment of various forms of bone marrow failure as well as malignant and nonmalignant disorders associated with the hemopoietic system.

Chapter 3 deals with the identification and characterization of a cDNA encoding human interleukin-3 and its expression in various systems. The molecular cloning of human IL-3 was accomplished by probe-hybridization screening of a human IL-3 cDNA library with a radiolabeled murine IL-3 cDNA probe. Generation of antibodies capable of immunospecific reaction with human IL-3 and large-scale production of human IL-3 in *Bacillus licheniformis* were performed in collaboration with the Research and Development department of Gist brocades, Delft, The Netherlands. Part of these collaborative studies are presented in chapter 3 as well. The availability of well-defined recombinant homogeneous human IL-3 prompted preclinical in vivo studies in human and nonhuman primates.

Limited, and in part inconsistent, effects of recombinant human IL-3 administered to rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*) suggested a functional species specificity. To enable preclinical studies in rhesus monkeys with homologous IL-3, the gene encoding rhesus monkey interleukin-3 (RhIL-3) was isolated as described in **chapter 4**. Expression of RhIL-3 genomic sequences in COS cells and the construction of a RhIL-3 cDNA are presented. The large-scale production of RhIL-3 in *Bacillus licheniformis*, required for *in vivo* studies was also developed in collaboration with the Research and Development department of Gist brocades.

The biological properties of recombinant human and rhesus monkey IL-3 produced by various organisms are described in **chapter 5**. These *in vitro* studies showed different stimulatory capacities between human and rhesus monkey IL-3 on rhesus monkey bone marrow cells. The impaired capacity of human IL-3 to support colony formation by rhesus monkey bone marrow cells have been further explored and both *in vitro* (see chapter 5) and *in vivo* (Van Gils, 1993) studies demonstrated a unidirectional species specificity of human IL-3 and suggested a rapid evolution of the IL-3 protein

Chapter 6 is directed at a further analysis of the molecular evolution of IL-3. Preliminary comparative studies between natural IL-3 variants from various species indicated differential substitution rates and highly variable distribution of synonymous (silent) and nonsynonymous (causing amino acid replacement) substitutions. Therefore, the IL-3 genes of chimpanzee, tamarin and marmoset

were identified to further explore the singular molecular evolution of IL-3. The established nucleotide as well as protein sequences of IL-3 from various species, i.e., mouse, rat, marmoset, tamarin, rhesus monkey, gibbon, chimpanzee and man were subjected to pairwise comparisons as described in chapter 6. Multiple sequence alignments of IL-3 coding regions and their encoded proteins showing a few conserved regions are presented and differential evolutionary rates among IL-3 species are discussed. Identification of regions implicated in IL-3 ligand binding to its cognate receptor and potential amino acid sequences determining species specificity and the overall IL-3 architecture were further investigated by mutational analysis as well as by analysis of constructed primate interspecies chimeric IL-3 proteins. Results of these structure-activity relationship studies, which were performed in collaboration with dr ir. L.C.J. Dorssers at the Molecular Biology department of the Dr. Daniel den Hoed Cancer Center, Rotterdam, The Netherlands, are discussed with reference to the molecular evolution of IL-3.

The general discussion of **chapter 7** is focussed on the implications of the species specificity on preclinical studies of IL-3 in human and nonhuman primates. Data on preclinical studies using homologous IL-3 in rhesus monkeys are briefly discussed. The nature of the species specificity with reference to the current knowledge of IL-3 and its cognate receptor and the molecular evolution of IL-3 and its receptor subunits are discussed. An attempt is made to place the regulatory function of IL-3 into perspective.

CHAPTER 2

MATERIALS AND METHODS

2.1 Molecular biology

2.1.1 Bacterial strains, plasmids and transformation procedures

The plasmids: pTZ18R/19R, pUC8/9, pUC18/19, M13mp18/19 and pSVL (Vieira and Messing, 1982; Messing, 1983) were purchased from Pharmacia and pGEM-7Zf(+/-) and pSP64/65 from Promega. Bacterial strains (all derived from *Escherichia coli*) used: HB101, DH1, C600hfl-, JM109, DH5α, ED8767, LE392. Transformation procedures were as described by Hanahan (1985) or according to the recommendations by the suppliers of competent cells (Epicurian Coli JM109, Stratagene Cloning Systems, California 92037; Subcloning efficiency *E. coli* DH5α competent cells, BRL). DNA modifying enzymes and restriction nucleases were generally obtained from Boehringer and GIBCO/BRL, unless otherwise indicated.

pL0 is an eukaryotic expression plasmid used to insert hIL-3 for expression in COS cells. The vector comprises: EcoRI (filled in)-PstI of pBR322 (1-755), PstI-*Ava*I of pBR329 (756-1849), *Ava*I-*Pvu*II adapter (1850-1868), *Pvu*II-*Hind*III (filled in) of SV40 promoter (1869-2211), *Pvu*II-*Bam*HI adapter containing the unique *Eco*RI site (2211-2251), *Mbo*I "splice fragment" of SV40 (2252-2861), *Bcl*I-*Bam*HI (filled in) "poly A fragment" of SV40 (2862-3098), *Pvu*II-*Hind*III promoter fragment of SV40 (3099-3440), *Hind*III-*Bam*HI "Eco gpt transcription unit" (3441-4501), *Mbo*I "splice fragment" of SV40 (4502-5111) and the *Bcl*I-*Bam*HI (filled in) "poly A fragment" of SV40 (5112-5348).

pLB4, the hIL-3 eukaryotic expression vector, was constructed by inserting hIL-3 cDNA into the unique *Eco*RI site of pL0 (Dorssers et al., 1987). The hIL-3 cDNA *Eco*RI fragment was retrieved by digesting clone D11 with *Hind*III and *Bgl*II, subsequent subcloning in pT1 (see below) and removed from this plasmid by partial digestion with *Eco*RI and purified by polyacrylamide gel electrophoresis.

pLH1 was constructed by deleting the 3'-terminal noncoding sequences between the *Ava*I site (pos. 541) and the *Xho*I site (pos. 856) in pLB4. The resulting sticky ends were filled in by the Klenow enzyme and fused with T4 ligase.

pT1 is a plasmid derived from pTZ18R whereby its multiple cloning site has been modified. The multiple cloning site of pT1 encompasses:5'- GGG AAT TCG AGC TCG ATA TCA AGC TTA GAT CTC CAG GGG GAT CCT CTA GAG TCG ACC TGC AGG CAT GCA AGC TGC ATA TGC AGC TTG -3'

pUC/hMulti is a pUC8 derived lacZ/hIL-3 bacterial expression vector. For the construction of this vector the following steps were performed. The pLH1 vector was digested with *Ava*II and the recessed ends were filled in with Klenow. Following ligation of a *Bgl*II linker (CAGATCTG) to the DNA, it was digested with *Bgl*II and *Bam*HI. The resulting hIL-3 fragment was purified and subcloned in the *Bgl*II site of pT1. Following sequence analysis of the resulting

pT-hIL-3, the *Bgl*II-*Eco*RV insert was isolated and subcloned into *Bam*HI and *Hind*II digested pUC8.

pUC/hMultiΔ1 and pUC/hMultiΔ2 were derived from plasmid, pUC/hMulti, lacking the coding region of the signal peptide (-19 to +2 aa) and the N-terminal -19 to +15 aa, respectively. The *Hind*II-*Hind*III fragment of pUC/hMulti was isolated and ligated to synthetic oligonucleotides, comprising the nucleotides 99-137 of hIL-3 and a 5'-terminal *Sal*I recognition site, and pTZ18R DNA digested with *Sal*I and *Hind*III. Following sequence analysis, a correct insert and an insert lacking the synthetic oligonucleotide which was found to be fused in frame to lacZ gene were transferred to pUC8 resulting in these fusion protein vectors.

pUR/hMulti 6 was derived from plasmid pUR 288 (Rüther and Müller-Hill, 1983). The *Sal*I-*Sma*I fragment was isolated from pT-hIL-3. The pUR 288 plasmid was digested with *Sal*I and *Hind*III. To construct the pUR/hMulti 6 the *Sal*I-*Sma*I hIL-3 fragment and the *Sal*I-*Hind*III (filled in with Klenow) were ligated.

pGB/RhIL-326 is the major prokaryotic RhIL-3 expression vector and was derived from the human IL-3 prokaryotic expression vector pGB/IL-326 (Persoon and Van Leen, 1990). The human IL-3 cDNA sequences (*Nde*I-*Hind*III fragment) of pGB/IL-326 were replaced by the RhIL-3 cDNA sequences resulting in pGB/RhIL-326 (Burger et al., 1990a). This vector contains an α-amylase signal peptide/mature RhIL-3 open reading frame placed downstream of the strong HpaII promoter and is followed by the α-amylase terminator. *B. licheniformis* strain T9 was thereupon used to express the RhIL-3 cDNA (Van Leen et al., 1991).The consequently secreted RhIL-3 protein contains 14 NH_2-terminal aa derived from the human IL-3.

pSVL/RhIL-3, the RhIL-3 eukaryotic expression vector (Burger et al., 1990a), was constructed by inserting the 2.2 kb SmaI-XhoI (filled in) genomic fragment of RhIL-3 into the SmaI site of the polylinker of pSVL (Pharmacia). The inserted fragment, comprising all RhIL-3 coding sequences, is under the control of the heterologous SV40 late promoter.

pCMV-4 (Andersson et al., 1989) was used to express the IL-3 coding sequences of chimpanzee (*Pan troglodytes*), marmoset (*Callithrix jacchus*) and tamarin (*Saquinus oedipus*). Genomic sequence containing the complete IL-3 coding sequences of these primates were obtained by PCR using IL-3 specific primers (Burger et al., 1994a,b; Dorssers et al., 1994).

2.1.2 Isolation of nucleic acids

Genomic DNA was isolated from mammalian cells according to standard procedures, including proteinase K digestion and phenol extraction (Bernards and Flavell, 1980; Maniatis et al., 1982).

Plasmid DNA was isolated according to standard procedures and, if appropriate, closed circular plasmid DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (Maniatis et al., 1982).
Total RNA was isolated following standard procedures (Favaloro et al., 1980) or according to a modification of this method using guanidinium isothiocyanate to homogenize the cells prior to RNA pelleting through a cesium chloride cushion (Maniatis et al., 1982). The resulting total RNA was subjected to chromatography on oligo-(dT)-cellulose (Maniatis et al., 1982) to separate it from nonpolyadenylated RNA (ribosomal and transport RNA) resulting in purified polyadenylated RNA (mRNA).
RF and single stranded bacteriophage DNA were isolated according to standard procedures (Maniatis et al., 1982; Messing, 1983). With respect to pTZ and pGEM derived plasmids, induction of single stranded DNA templates was achieved following infection of bacterial cells with an appropriate helper phage (M13K07) as described (pGEM Single Strand System, Technical Manual, Promega).

2.1.3 Nucleic acids probes

The complete murine IL-3 cDNA was isolated from plasmid pL101 (*Pst*I insert, see chapter 3) and radiolabeled according to the random primers procedure (Feinberg and Vogelstein, 1983) with [α-^{32}P] dCTP. Radiolabeled RNA molecules, transcribed from the *Hind* III-*Xba*I fragment of murine IL-3 cDNA, were prepared using the SP6 promoter present in the pSP64 plasmid (Promega). The resulting murine IL-3 SP6 RNA probe and the complete murine IL-3 cDNA probe were used to screen the human leukocyte cDNA library for the presence of human IL-3 (Dorssers et al., 1987).

Screening of the rhesus monkey genomic DNA library was done with a human IL-3 cDNA probe, devoid of the 3' noncoding region (nt 541-856) containing repetitive sequences. This probe was isolated from plasmid pLB4 (Dorssers et al., 1987) and radiolabeled with the help of random primers and [α-^{32}P] dCTP.

All probes used for Southern blot analyses, including the complete hIL-3 cDNA (nt 1-910), 5'-fragment (nt 1-253) as well as 3'-fragment (nt 411-910) of hIL-3 cDNA, hIL-3 cDNA lacking 3' noncoding region (nt 1-541) were radiolabeled either with [α-^{32}P] dCTP or [α-^{32}P] dATP (110TBq/mmol; Amersham, Life Science Products) with the help of random primers (Feinberg and Vogelstein, 1983).

2.1.4 Construction and screening of nucleic acids libraries

2.1.4.1 Murine WEHI-3B cells cDNA library

WEHI-3B cells, constitutively producing SAF which appeared to be identical to mIL-3 (Dorssers et al., 1984), were collected and mRNA was isolated using the Nonidet-P40/vanadyl-ribonucleoside complexes cell lysis and oligo (dT)-cellulose chromatography selection protocol (Maniatis et al., 1982). The resulting polyadenylated RNA was fractionated on a linear sucrose gradient. From the fractions containing mIL-3 mRNA, identified using micoinjection into *Xenopus laevis* oocytes and subsequent testing of the translation products, 3 µg poly A+ mRNA was used in the oligo (dT)-primed cDNA synthesis reactions (Gubler and Hoffman, 1983). Single stranded cDNA was converted into double stranded (ds) cDNA by *E. coli* RNase H, *E. coli* DNA polymerase I and T4-DNA ligase (all enzymes supplied by Boehringer, Mannheim). Approximately 1 µg of the constructed cDNA was tailed with dC residues, without prior size-selection, and annealed to dG-tailed pUC-9 vector DNA (Pharmacia). Resulting plasmids were propagated in the *Escherichia coli* (*E. coli*) strain DH1. Nitrocellulose filters (Millipore HAWP) containing replicas of bacterial colonies were prepared according to established procedures (Maniatis et al., 1982). Two selected synthetic oligonucleotides (A: nt 30-48 and B: nt 477-498), based on the reported murine IL-3 sequence (Fung et al., 1984), were used as hybridization probes for screening this murine WEHI-3B cells cDNA library. The conditions for the identification of recombinant clones by *in situ* hybridization of bacterial colonies was according to standard procedures (Maniatis et al., 1982).

2.1.4.2 Human leukocyte cDNA library

Polyadenylated mRNA was extracted from stimulated human leukocytes (see section 2.1.1 of this chapter), using the guanidinium isothiocyanate/CsCl and the oligo (dT)-cellulose chromatography method (Maniatis et al., 1982). The poly A+ mRNA was reversed transcribed into complementary DNA (cDNA) according to Gubler and Hoffman (1983). The resulting cDNA was methylated with *Eco*RI methylase and ligated to *Eco*RI linkers. The cDNA was subsequently digested with *Eco*RI, the excess of linkers removed by Sepharose CL-4B chromatography (Verweij et al., 1986), and inserted into the *Eco*RI site of λgt 10 phage DNA (Gigapack, Vector Cloning Systems). *In vitro* packaged phage DNA was used to infect the *E. coli* strain C600 hfl- and plated onto 15 cm agarose plates (Huynh et al., 1985; Maniatis et al., 1982). Nitrocellulose filters containing replicas of bacteriophage plaques were prepared according to established procedures (Maniatis et al., 1982). Hybridization screening with the murine IL-3 cDNA probes was carried out at 55°C (RNA) or 50°C (DNA) in 5 x SSPE, 1 x Denhardt, 0.5% SDS, in the presence of 100 µg/ml of salmon

sperm DNA for 16-20 h (Meinkoth and Wahl, 1984). Filters were washed at 55°C in 2 x SSC and 0.1% SDS and autoradiographed with Kodak XAR films.

2.1.4.3 Rhesus monkey genomic DNA library

Lambda Charon 40 phages containing rhesus monkey genomic DNA partially digested with *Eco*RI, kindly provided by Dr. Jerry L. Slightom (The Upjohn Company, Kalamazoo, Michigan 49001, USA.), were used to infect the recA-*Escherichia coli* host ED8767. Infectants were subsequently seeded onto 15-cm agarose plates. Nitrocellulose filters containing replicas of the recombinant bacteriophage plaques were prepared according to standard procedures (Maniatis et al., 1982). Hybridization of the filters with the human IL-3 cDNA probe (nt 1-541), lacking the 3'-untranslated repetitive sequences, was carried out at 65°C in 50 mM Tris, 1 M NaCl, 10 x Denhardt, 0.5% SDS, 0.1% PP_i in the presence of 100 µg/ml of herring sperm DNA for 16-20 h. Filters were washed at 65°C in 2 x SSC, 0.1% SDS and 0.1% PP_i and autoradiographed with Kodak XAR films.

2.1.4.4 RhIL-3 transfected COS cell cDNA library

To enable expression of RhIL-3 in prokaryotes a cDNA was constructed from mRNA extracted from COS cells, which were transfected with pSVL/RhIL-3. Isolation of cytoplasmic RNA (Favaloro et al., 1980) was carried out 48 hours after transfection followed by isolation of poly A+ mRNA using an oligo (dT)-cellulose column (Maniatis et al., 1982). The first strand reaction of cDNA synthesis was performed on 2 µg of poly A+ mRNA using oligo (dT) as primer (Selten et al., 1986). After completion of the reaction, EDTA was added, a chloroform extraction was carried out and the cDNA reaction products were precipitated with ethanol. After dissolving the pellet in distilled water, half of the single stranded cDNA was transferred to an eppendorf tube containing oligonucleotides designed for RhIL-3 cDNA cloning into a *Bacillus* expression vector. The first oligonucleotide has the sequence 5'-TGC GAC GTG GAT CCT GCA GCA GCG GCG GCT CCC ATG ACC CAG ACA ACG-3' and contains *Bam*HI and *Pst*I restriction sites and the information for the 3' part of the *B. licheniformis* alpha-amylase signal peptide precisely fused to the mature RhIL-3 sequence. The second oligonucleotide has the sequence 5'-GGA TCC GAG CTC CCC-3' and is complementary to the 3' end of mRNAs synthesized from pSVL constructs. After completion of the polymerase chain reaction (PCR) in which 25 cycles (2 min 94°C, 2 min 55°C and 3 min 72°C) were carried out according to the recommendations of the supplier of Taq-polymerase (Cetus), the reaction products were digested with *Bam*HI and *Xho*I and the resulting fragments were inserted into pGEM-7Zf(+) (Promega). The resulting plasmids were propagated in the *E. coli* strain JM109 (BRL). Without prior hybridization selection,

individual transformants were directly subjected to DNA sequence analysis.

The insert of a correct clone was excised from the pGEM-7Zf(+) vector using *Pst*I and *Xho*I and transferred to the *Pst*I-*Xho*I digested *E. coli* vector pGB/IL-340 (Dorssers and Van Leen, 1991), resulting in the *E. coli* vector pGB/RhIL-340. For expression in *B. licheniformis* the *Nde*I-*Hind*III fragment from pGB/RhIL-340 was used to replace the *Nde*I-*Hind*III fragment of pGB/IL-326 (Persoon and Van Leen, 1990). In the resulting vector pGB/RhIL-326 the alpha-amylase signal peptide/mature RhIL-3 open reading frame is placed downstream of the strong *Hpa*II promoter and is followed by the amylase terminator. *B. licheniformis* strain T9 was thereupon transformed with plasmid pGB/RhIL-326 to express the RhIL-3 cDNA.

2.1.4.5 Chimpanzee, marmoset and tamarin IL-3 genes

Chimpanzee and marmoset chromosomal DNA were extracted from mononucleated peripheral blood cells derived from monkeys held at the Primate Center in Rijswijk, The Netherlands. Tamarin genomic DNA was isolated from the Epstein-Barr Virus (EBV) transformed B-cell line B95-8 (Miller et al., 1972; ATCC CRL1612). The PCR technique was applied to amplify the IL-3 coding regions of chimpanzee, marmoset and tamarin. IL-3 specific oligonucleotide primers, corresponding to identical or highly conserved regions between the human and rhesus monkey IL-3 genes, were selected for the amplification reactions (Burger et al., 1994a,b; Dorssers et al., 1994). Amplified fragments, resulting from at least two independently performed PCR reactions, were isolated using traditional cloning techniques and the nucleotide sequences were determined in both directions. The cDNA sequences were obtained by RT-PCR following transient expression of genomic constructs in COS cells (Burger et al., 1994a).

2.1.5 Nucleotide sequence determination

The various cDNA inserts, plaque-purified or colony-purified in the case of the mIL-3 cDNA, of positive clones were purified on acrylamide gel and subcloned into suitable filamentous phagemids such as M13mp18/19, pTZ18R/19R (Pharmacia) or pGEM-7Zf(+/-) (Promega).

The various human and murine IL-3 cDNAs were subcloned into M13mp18/19 or pTZ18R/19R and single stranded DNA was prepared and sequenced in both directions according to the chain-termination method (Sanger et al., 1977) or according to instruction manuals supplied by BRL (1985, BRL M13 Cloning/Dideoxy Sequencing Instruction Manual) and Pharmacia P-L Biochemicals (Manual for the M13 Cloning/Sequencing System).

The 4.1 kb *Eco*RI fragment, containing the complete RhIL-3 gene, was digested with *Ava*I and *Bam*HI. Blunt ends were created by filling in the

restriction enzyme termini with the Large Fragment of DNA Polymerase I (Klenow Fragment) and the fragments were subcloned into the sequence vector pTZ18R (Pharmacia). Using the KiloBase Sequencing System (Bethesda Research Laboratories), fragments were sequenced in both directions and oligonucleotides were synthesized, complementary to the established sequences to serve as internal primers for sequencing over the restriction sites used for subcloning.

From several individual pGEM-7Zf(+) transformants, containing rhesus monkey IL-3 cDNA, the nucleotide sequence was established using the KiloBase Sequencing System (Bethesda Research Laboratories). All the sequence data obtained as described in this section were analyzed using the Microgenie computer program (Queen and Korn, 1984).

All the sequence data have been deposited at the EMBL Data Library and assigned the following accession numbers: X74875 (*P.troglodytes* IL-3, exons 1 and 2), X74876 (*P. troglodytes* IL-3, exons 3, 4 and 5), X74877 (*C. jacchus* IL-3 mRNA), X74878 (*S. oedipus* IL-3, exons 1 and 2), and X74879 (S. oedipus IL-3, exons 3, 4 and 5). The other nucleotide and amino acid sequence data, i.e., mouse (Fung et al. 1984; accession no:K01850), rat (Cohen et al. 1986; X03846 and X03914), rhesus monkey (Burger et al. 1990b; no: X51890), gibbon (Yang et al. 1986; accession no: M14744)) and man (Dorssers et al. 1987; accession no: M17115) were obtained either from the Genbank or the EMBL data library.

2.1.6 Transfection procedures of mammalian cells

Closed circular plasmid DNA (pLB4 or pLH1), purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (Maniatis et al., 1982) was transfected to COS 1 cells (Gluzman, 1981) using the calcium phosphate co-precipitation method (Graham and Van der Eb, 1973). Cells were cultured for 48-72 hours in enriched Dulbecco's medium (Wagemaker and Visser, 1980) containing 10% fetal calf serum. COS conditioned medium was harvested, filtered (.22 μm Millipore Filter) and used in the bioassays as described below. Transfection procedures to establish stable cell lines expressing hIL-3 (C127 and CHO cells) were according to the procedure described above and are described in more detail elsewhere (Dorssers et al., 1987b; Persoon and Van Leen, 1990; Van Leen et al., 1990/1991). Plasmid pSVL/RhIL-3, purified as described above, was transfected to COS cells using the DEAE-dextran/chloroquine method (Luthman and Magnusson, 1983). Eukaryotic expression constructs carrying primate IL-3 genes were purified using the Qiagen-kit (Diagen GmbH, Düsseldorf, FRG) and introduced into COS cells using the Lipofectin Reagent (GIBCO BRL) in serum-free medium for 4-16 hours (Burger et al., 1994a; Dorrsers et al., 1994).

2.1.7 Chromosomal analysis

Chromosomal DNA was extracted from mammalian cells according to standard procedures (Bernards and Flavell, 1980). Approximately 15 µg DNA of 20 somatic hybrid cell lines between human and rodent cells was digested with *Bam*HI or *Eco*RI prior to size fractionation on 0.6% agarose gels in Tris/Borate buffer. Following electrophoresis, DNA fragments were transferred onto nylon membranes (GeneScreen Plus, NEN Research Products Boston) and hybridized to random-primer labeled hIL-3 cDNA (nt 1-541 fragment of clone D11). The chromosomal constitution of the hybrid cell lines was deduced from R-banded metaphase spreads using acridine orange (Geurts van Kessel et al., 1983).

2.2 Cell cultures

2.2.1 Human peripheral blood leukocytes

Blood samples of healthy volunteers were collected after obtaining informed consent. Human peripheral blood leukocytes were purified by Ficoll gradient (Nycomed, Oslo, Norway) centrifugation and cultured for 24 h in enriched Dulbecco's medium (Wagemaker and Visser, 1986) containing 10% FCS (v/v), 10 µg concanavalin A (ConA; Pharmacia) and 5 ng 12-0-tetradecanoylphorbol-13-acetate (TPA; Sigma) per ml.

2.2.2 AML-193 assay

An established IL-3 dependent human AML cell line (AML-193) was used to measure IL-3 activity (Lange et al., 1987). AML-193 cells, grown in the presence of recombinant hIL-3 (10 µg/l), were collected, washed and resuspended in modified Dulbecco's medium (Wagemaker and Visser, 1980) supplemented with 4.8 mg/l human transferrin (Behring) and 5 mg/l Insulin (Gibco). After culturing 2 x 10^4 cells / 200 µl / well for 4 days in microtiter plates, cells were radiolabeled with [2-^{14}C]Thymidine (0.08 µCi/well) for 1 day. Cells were harvested and incorporation was measured with the liquid scintillation analyzer and expressed as counts per minute (CPM). The amount of IL-3 per ml, required to give half-maximal response, is designated as 1 unit.

2.2.3 Proliferation and colony formation assay using AML blasts

The effects of IL-3 on colony formation by acute myeloid leukemia (AML) blast cells were determined in the "AML colony formation" assay. A modified version of the "phytohaemagglutinin leukocyte feeder" (PHA LF) system (Swart and Löwenberg, 1984) in which the leucocyte feeder was replaced by hIL-3 preparations (COS[pLB4] CM) permitted the assessment of the colony-

stimulating activity of hIL-3 preparations. All experiments were preformed in triplicate. All AML patients had given informed consent to donate bone marrow. The leukemic cells from individual adult AML patients were separated from the bone marrow using a BSA density gradient (Swart and Löwenberg, 1984). T lymphocytes were removed by E rosette sedimentation as described elsewhere (Gerritsen, 1989). The resulting AML cell preparations contained >90% blasts. Following this procedure the cells were cryopreserved in 7.5% DMSO and 20 % fetal calf serum (FCS; Flow laboratories, Isrike, UK) as described by Delwel et al. (1986). The "AML proliferation" assay was used to measure DNA synthesis of AML blasts. DNA synthesis was assayed by tritiated thymidine incorporation, essentially as described by Delwel et al. (1987).

2.2.4 Human bone marrow cultures

Human bone marrow was obtained by posterior iliac crest puncture from hematologically normal adults, who had given their informed consent. The mononucleated cells were separated by density-gradient centrifugation over Ficoll-Isopaque (1.077g/cm^3; Nycomed, Oslo Norway) as described (Bot et al., 1988) and, if appropriate, cryopreserved till further use as previously described (Gerritsen et al., 1988). Enrichment of progenitor cells was achieved following depletion of T lymphocytes and accessory cells by complement mediated lysis using the monoclonal antibodies OKT-3 (CD3; Ortho CO., Raritan, NJ, U.S.A.) and Vim2 (Majdic et al., 1984) and selection on the basis of CD 34 expression according to established procedures (Bot et al., 1988). If appropriate, the cultures were supplemented with recombinant human erythropoietin ([Epo, 1 U/ml] Amgen Biological, Thousand Oaks, California) and scored according to established criteria (Bot et al., 1988) for erythroid, granulocytic and mixed colonies.

Various human bone marrow assays such as the proliferation-, colony formation- and liquid culture assay were used to assess the biological activity of hIL-3 preparations as discussed in chapter 5. The "hBM proliferation" assay determines the stimulatory capacity of IL-3 preparations by measuring DNA synthesis. The latter was measured either by ^{3}H-thymidine incorporation as described (Touw, 1986) or [2-^{14}C] thymidine incorporation. The "hBM colony formation" assay determines the capacity of IL-3 preparations to support colony growth by bone marrow progenitor cells in methylcellulose as described (Bot et al., 1989). The "hBM liquid culture" assay determines the capacity of IL-3 preparations to support the production of GM-CSF responsive clonogenic cells in liquid cultures. Bone marrow cells (3 x 10^5 per ml) were cultured in the presence of graded concentrations of IL-3 in enriched Dulbecco's medium (Merchav and Wagemaker, 1984) supplemented with 1.5 % BSA (Sigma, fraction V) and 5% FCS (Biochrom) for 6 (human) or 7 (Rhesus monkey) days at 37^{0}C in a saturated humidified atmosphere of 10% CO_2 in air. Following

incubation with IL-3, the cells were washed and the production of GM-CSF responsive progenitor cells was assayed in methylcellulose (Methocel A4M Premium grade, Dow chemical) cultures as previously described. Recombinant human GM-CSF (Glaxo Inc., Genève, Switzerland) was used at supraoptimal concentrations (5 nanogram per ml) to support colony growth. Colonies were scored on day 10, according to established criteria (Bot et al., 1988), using an inverted microscope (Zeiss) at a 30-fold magnification.

2.2.5 Rhesus monkey bone marrow cultures

Rhesus monkey bone marrow cells were obtained and subjected to discontinuous density gradient centrifugation to concentrate stem cells and progenitor cells as previously described (Wagemaker et al., 1982). T lymphocytes were removed from the resulting stem cell concentrates by E-rosette sedimentation (Gerritsen et al., 1988). The T lymphocyte depleted stem cell concentrates were cryopreserved (Schaeffer et al., 1972) till further use. After thawing and removal of DMSO by step-wise dilution, the cells were used in various rhesus monkey bone marrow culture systems, analogous to the human bone marrow cultures described in the previous section .

Further enrichment of progenitor cells was achieved by two distinct procedures. In both cases the cells were first labelled with the monoclonal antibody MO1 (Coulter Immunology). MO1 positive cells were removed by immunomagnetic beads separation (Wielenga, 1990) to deplete the suspension of accessory cells influencing colony growth. The resulting MO1 depleted suspension was further labelled with anti-HLA-DR (Becton Dickinson, 1ml/5 x 107 cells), an antigen known to be densely present on stem cells and progenitor cells (Gerritsen et al., 1988). HLA-DR positive cells were conjugated to protein-A, that was covalently bound to immunomagnetic beads and than removed from the suspension by a magnet. The cells were eluted from the beads by excess immunoglobulin in normal bovine serum. The resulting T lymphocyte depleted, MO1-/DR+ stem cell concentrates were used for IL-3 stimulated cultures at concentration as indicated in the figure legends. Since the IL-3 response is known to be impaired in the absence of accessory cells (Bot et al., 1988), appropriate numbers of MO1 positive cells, prepared by the same immunomagnetic beads separation method, were added to the cultures as indicated in the figure legends. Alternatively, the MO1 depleted cell suspension was labeled with the monoclonal antibody ICH3 as described (Wielenga, 1990). ICH3 recognizes an epitope of the CD34 antigen and was a gift of Dr R. Levinsky (Institute of Child Health, London, UK). Both procedures result in highly enriched rhesus monkey bone marrow progenitor cell populations, which were used in comparable assays as described above for human bone marrow. The methylcellulose cultures and scoring of hemopoietic colonies were done according to methods previously described (Merchav and Wagemaker, 1984;

Bot et al., 1988; Wielenga, 1990). Hemopoietic colonies were scored after two weeks of culture.

2.3 Protein chemistry

2.3.1 SDS-polyacrylamide gel electrophoresis and Western blot analysis

SDS-polyacrylamide gel electrophoresis was performed essentially as described (Laemmli, 1970; Protean II Slab Cell system, Bio-Rad) and the PhastSystem™ (Pharmacia) was used to detect IL-3 proteins in chromatography separated fractions. Following size-selection of the proteins by SDS acrylamide gel electrophoresis (13.5% unless otherwise indicated) the proteins were transferred to nitrocellulose membranes using the semi-dry transfer technique (LKB 2117 multiphorII, 0.8 mA/cm2 for 1-2 hours, Transfer buffer: 48 mM Tris, 39 mM Glycine, 0.0375% SDS [w/v] and 20% methanol [v/v]) The nitrocellulose membranes were blocked for 4-6 hours at room temperature in 10 mM Tris-Hcl (pH 7.6), 350 mM NaCl, 0.1mM PMSF, 0.1% (w/v) NaN_3, 0.3% (w/v) BSA. The membranes were incubated (overnight with gentle agitation) with the primary antibody, polyclonal rabbit anti-hIL-3 (using 1:5,000 dilutions) or monoclonal mouse anti-hIL-3 (using 1:500 dilutions) in PBT (PBS supplemented with 0.05% [w/v] Tween 20 and 0.5% [w/v] BSA), followed by three 10-min washes with PBT. The primary polyclonal as well as monoclonal antibodies were prepared as described elsewhere (Dorssers et al., 1987b). Following incubation with the primary antibody, membranes were incubated with second antibody (2^0 Ab; biotinylated donkey anti rabbit/mouse IgG (dependent on the primary antibody; 1; 1000 dilutions, Amersham), followed by three 10-min wash steps in PBT. The 2^0 Ab was conjugated to streptavidin-horseradishperoxidase (SA-HRP; 1: 1000 dilutions, Amersham) by 60-min incubation at room temperature. Membranes treated with SA-HRP conjugates were incubated in 4-CN solution (0.5 mg/ml 4-chloro-1-naphthol [Sigma], 15% [v/v] Methanol and 0.05% [v/v] H_2O_2) in PBS for 30 min ar room temperature with agitation protected from light. IL-3 specific signals on western blots were quantitated by densitometric scanning of the membranes. Alternatively, IL-3 immune complexes with peroxidase-coupled rabbit antimouse immunoglobulin antibody were visualized by enhanced chemoluminescence (ECL) substrate reaction (Amersham Corp.) as described elsewhere (Dorssers et al., 1991; Burger et al., 1994a; Dorssers et al., 1994).

2.3.2 Protein purification steps

Various human IL-3 fusion proteins were produced by *E.* coli strain JM109. Bacteria were grown in LB medium (Gibco) containing 50 µg/ml of ampicillin

at 37°C until an optical density (550 nm) of 0.5 followed by supplementing IPTG (Pharmacia) to a final concentration of 1 mM. Incubation was continued for 3-4 hours before bacteria were collected by centrifugation and disrupted by sonication in buffer containing 0.1 M Tris/HCL, pH 8.0; 5 mM EDTA; 0.2% Nonidet P40 (NP-40) and 1 mM phenylmethylsulfonylfluoride (PMSF). Soluble components were separated from the inclusion bodies by centrifugation at 20,000 *g* for 30 min. The pellet was re-extracted with 0.5% NP-40 buffer and finally solubilized in buffer containing 8 M urea; 0.1 M Tris/HCL, pH 8.0 and 5 mM dithiothreitol (DTT).

Purification procedures for hIL-3, produced by a wide range of host cells (see chapter 3), are described elsewhere (Dorssers et al., 1987a,b; Persoon and Van Leen, 1990; Van Leen et al., 1990; Dorssers et al., 1991).

Purification of RhIL-3 expressed by B. licheniformis was performed as previously described in detail for hIL-3 (Persoon and Van Leen, 1990; Van Leen et al., 1990) with the exception that for the ion-exchange chromatography step DEAE Sepharose Fast Flow (Pharmacia) was used instead of Fractogel TSK DEAE-650. Briefly, *B. licheniformis* supernatant, containing the RhIL-3 polypeptide, was applied onto a TSK Butyl-650(C) hydrophobic interaction chromatography column (Merck). Bound proteins including RhIL-3 were eluted by using a steep ammonium sulphate gradient (1-0 M). Fractions containing RhIL-3 were pooled and the protein was precipitated using 70% ammonium sulphate. The precipitate was dissolved in anion-exchange chromatography starting buffer and after dialysis against the same buffer fractionated by DEAE Sepharose Fast Flow. The unbound RhIL-3 was collected and tested for its purity by polyacrylamide gel electrophoresis according to standard procedures and western blot analysis using polyclonal and monoclonal antibodies (Dorssers et al, 1987b) directed against the hIL-3 polypeptide.

Purification methods for chimpanzee, marmoset and tamarin IL-3 proteins from either eukaryotic or prokaryotic source were in principal according to the procedures as described above. Slightly modified methods are detailed elsewhere (Dorssers et al., 1991; Dorssers et al., 1994; Burger et al., 1994a).

2.4 Evolutionary analyses

The "Clustal" method of Higgins and Sharp (1988) was used to align the IL-3 proteins. This method is based on the iterative approach, i.e., the sequences are progressively aligned using the results of pairwise alignments. Prior to the multiple alignment, the method will construct a phylogenetic tree or dendrogram. The order of branching in the tree will, eventually, determine the ranking order of the pairwise alignments in order to construct the multiple alignment. The conserved intron/exon junctions of IL-3 species were used to verify whether the computer introduced gaps were located correctly. The protein alignment served as basis for the nucleotide sequence alignment.

We used the method of Li et al. (1985) to estimate the numbers of substitutions per synonymous site (K_S) and per nonsynonymous site (K_A) between two genes. This method includes corrections for multiple hits at one site (superimposed substitutions) and takes into account the differences in substitution rates between transversions (purine↔pyrimidine) and transitions (C↔T or A↔G) and the relative likelihood of codon interchanges. We are aware of the recently presented modified version (Li et al. 1993), which gave similar estimates for the K_A and somewhat lower estimates for the K_S. Since we highlight the accumulation of nonsynonymous substitutions and the unusually low K_S/K_A ratios, we have used the earlier version of the method for the present study.

Phylogenetic trees from distance matrices of homologous IL-3 sequences were constructed according to the program FITCH, a computer algorithm (PHYLIP package, manual version 3.3, 1990), developed by Felsenstein J, University of Washington, Seattle, Washington. This method, based on the approach of Fitch and Margoliash (1967), includes corrections for unequal rates of evolution and does not permit negative branch lengths.

2.5 Statistical analyses

With respect to the colony formation assays, standard deviations were calculated on the assumption that crude colony counts are Poisson distributed (Blackett, 1974). In all other cases standard deviations (SD) and standard errors of mean (SE) were determined according to the standard formula (Booster and van Kampen, 1977).

CHAPTER 3

MOLECULAR CLONING OF A HUMAN INTERLEUKIN-3 cDNA

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Wagemaker, G., H. Burger, F.C.J.M. van Gils, R.W. van Leen and J.J. Wielenga. Interleukin-3. Biotherapy. 2: 337-345, 1990.

Van Leen , R.W., J.G. Bakhuis, R.F.W.C. van Beckhoven, H. Burger, L.C.J. Dorssers, R.W.J. Hommes, P.J. Lemson, B. Noordam, N.L.M. Persoon and G. Wagemaker. Production of human interleukin-3 using industrial microorganisms. Biotechnology. 9: 47-52, 1991.

3.1 Introduction

Interleukin-3 (IL-3) has for some time been recognized as a multi-lineage hemopoietic growth factor in mice. Along with the molecular cloning of murine IL-3 (mIL-3), most genes encoding murine colony-stimulating factors, including G-CSF, GM-CSF and erythropoietin, have been identified as described in chapter 1. The human genes encoding M-CSF, G-CSF, GM-CSF and erythropoietin have been identified and their products were shown to be functionally related to the murine homologs. Several investigators, including our own group, demonstrated that homologous IL-3 sequences could not be identified in human genomic DNA through annealing with a mIL-3 cDNA probe. Apparently, the human gene has diverged rapidly from the mIL-3 gene or lost its function during primate evolution as speculated by several investigators (Emerson et al., 1985; Cohen et al., 1986; Kindler et al., 1986). However, appropriately stimulated cultures of human leukocytes are a suitable source for human HGFs, demonstrated by stimulation of *in vitro* colony formation of normal human hemopoietic progenitor cells (Fausner and Messner, 1978) and by the capacity to support colony formation of blast progenitor cells in acute myeloid leukemia (Löwenberg et al., 1982; Buick et al., 1979). Furthermore, the stimulated human leukocytes were found to produce a factor which stimulates the proliferation of murine CFU-S *in vitro* (Wagemaker and Peters, 1978) analogous to murine IL-3 (SAF). These observation suggested the existence of a human HGF exhibiting biological properties functionally related to mIL-3. It was conceivable that this factor represented the human homolog of murine IL-3.

In order to identify the gene encoding the postulated human HGF, three alternative strategies were considered: i) probe-hybridization screening of a cDNA library containing the postulated HGF, ii) probe-hybridization screening of a genomic library containing DNA sequences of the entire human genome, iii) identification by functional expression cloning. Probe-hybridization screening relies on the availability of a suitable nucleic acid probe as well as a library representing the gene or cDNA encoding the desirable protein. With respect to a suitable nucleic acid probe, mIL-3 cDNA seemed to be the only potential candidate for probe-hybridization screening. However, it is noteworthy that Southern blot analysis of human genomic DNA did not allow detection of human homologous sequences using mIL-3 cDNA. Nevertheless, this murine IL-3 probe was supposed to be applicable to detect homologous sequences in human libraries since individual genes are significantly amplified by the bacterial host. A genomic library is in most cases already available but is less suitable for functional expression cloning, whereas a cDNA library has to be constructed for every mRNA source. A cDNA library can initially be used for probe-hybridization screening, and in case the method fails, the library can subsequently be used for functional expression cloning. An orientation-specific

cDNA cloning strategy had been developed in our group (Dorssers and Postmes, 1987) to simplify the functional expression cloning procedure. This strategy is considered in case probe-hybridization screening would not allow for the identification of human IL-3. Based on these considerations, a cDNA library was constructed using polyadenylated RNA extracted from appropriately stimulated human peripheral leukocytes. The λgt10 vector instead of a plasmid vector was used to take advantage of the high efficiency and reproducibility of *in vitro* packaging of lambda DNA as a method for introducing DNA sequences into bacteria such as *E. coli*. Prior to the screening of this human cDNA library, mIL-3 cDNA was isolated and subsequently used as a nucleic acid probe to screen the human cDNA library.

The retrieval of a hIL-3 cDNA, identified by exploiting the rather high degree of sequence homology within the 3'-untranslated region of the mouse and human cDNAs, is described in this chapter. Furthermore, the large-scale production of hIL-3 and the preparation of antibodies directed against the hIL-3 polypeptide will be presented in this chapter as well.

3.2 Molecular cloning of murine IL-3 cDNA

Murine stem cell activating factor (SAF), purified in our group from conditioned medium of the myelomonocytic cell line WEHI-3, and recombinant murine IL-3 were shown to exert identical biological activities. Moreover, the identity of these activities has been demonstrated using antibodies directed against murine IL-3 (Dorssers et al., 1984). Injection of polyadenylated mRNA, extracted from WEHI-3 cells, into *Xenopus laevis* oocytes resulted in detectable mIL-3 in the culture medium. This *in vivo* translation system allowed the isolation of a mIL-3 enriched mRNA population on sucrose gradients, required for the construction of a murine cDNA library as described in chapter 2.

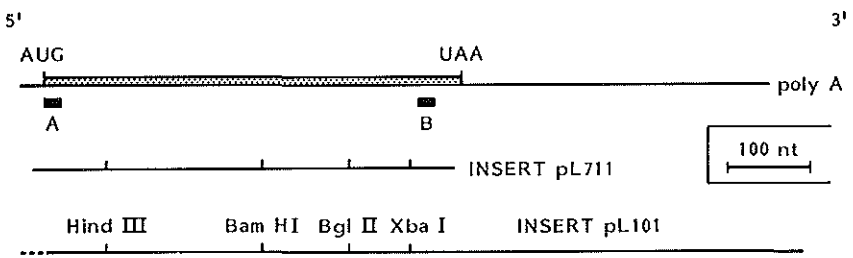


Fig. 3.1 Schematic representation of mIL-3 (SAF) mRNA. Restriction maps of murine IL-3 cDNA clones are shown. The positions of the oligonucleotide probes A (nt 30-48) and B (nt 477-498) are indicated. The initiation and termination codons are based on the sequence as reported by Fung et al. (1984).

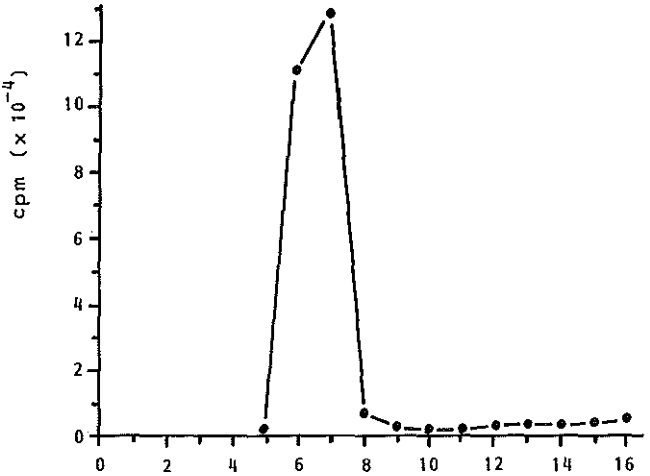
To accomplish the isolation of mIL-3 cDNA, approximately 30,000 colonies were hybridized with selected oligonucleotides (Fig. 3.1) based on the published mIL-3 sequence (Fung et al., 1984; Yokota et al., 1984). Two cDNA clones were identified and restriction enzyme mapping as well as determination of the nucleotide sequence demonstrated that these clones represent mIL-3. The IL-3 cDNA insert of pL101 contained the complete murine IL-3 coding information. Therefore, a purified and radiolabeled insert of this mIL-3 cDNA clone was used as a nucleic acid probe to screen human genomic and cDNA libraries.

3.3 Construction of a human cDNA library

Human peripheral leukocytes were cultured in the presence of the phorbol esther TPA and the lectin ConA for 24 hours to elevate the level of transcripts encoding several HGFs. Induction of growth factor synthesis was found to be optimal using these conditions reflected by the presence of murine stem cell activating factor (SAF) in this human leukocyte conditioned medium (HLCM). Furthermore, HLCM was shown to stimulate murine IL-3 dependent myeloid cells (DA-1). Polyadenylated mRNA, extracted and isolated from these stimulated leukocytes, was reversed transcribed into complementary DNA (cDNA), as described in chapter 2. The resulting cDNA was methylated with *Eco*RI methylase to protect internal *Eco*RI sites and ligated to *Eco*RI linkers. Following digestion with *Eco*RI, the excess of linkers was removed by Sepharose CL-4B chromatography ([Fig. 3.2], Verweij et al., 1986). As shown in Fig. 3.2 the length of the generated human cDNA recovered in the void volume of the column ranged from 400 till approximately 6.000 base pairs (bp). A cDNA library in the λgt10 phage, constructed as described in chapter 2, was hybridized with human IL-2 (hIL-2) sequences and approximately 0.1 % of the clones appeared to contain cDNA inserts homologous to hIL-2. This incidence is in agreement with its expected expression in stimulated leukocytes (Clark et al., 1984), indicating the efficiency of the procedure.

3.4 Isolation and identification of a human IL-3 cDNA

The mIL-3 cDNA insert of pL101 was purified on polyacrylamide gel and radiolabeled, as described in chapter 2, to function as nucleic acids probe for hybridization to the constructed human leukocyte cDNA library. The latter was screened either with radiolabeled SP6-transcribed RNA derived from the *Hind*III-*Xba*I fragment of the mIL-3 cDNA insert of pL101 or with the complete mIL-3 cDNA insert of pL101 (Fig. 3.1). The RNA probe lacks the A + T rich sequences present in the 3' non-coding region of the murine IL-3 gene (Miyatake et al., 1985), which, as turned out later, are also present in the 3' end of many other HGFs (Kaushansky,1987).


cpm (x 10^-4)
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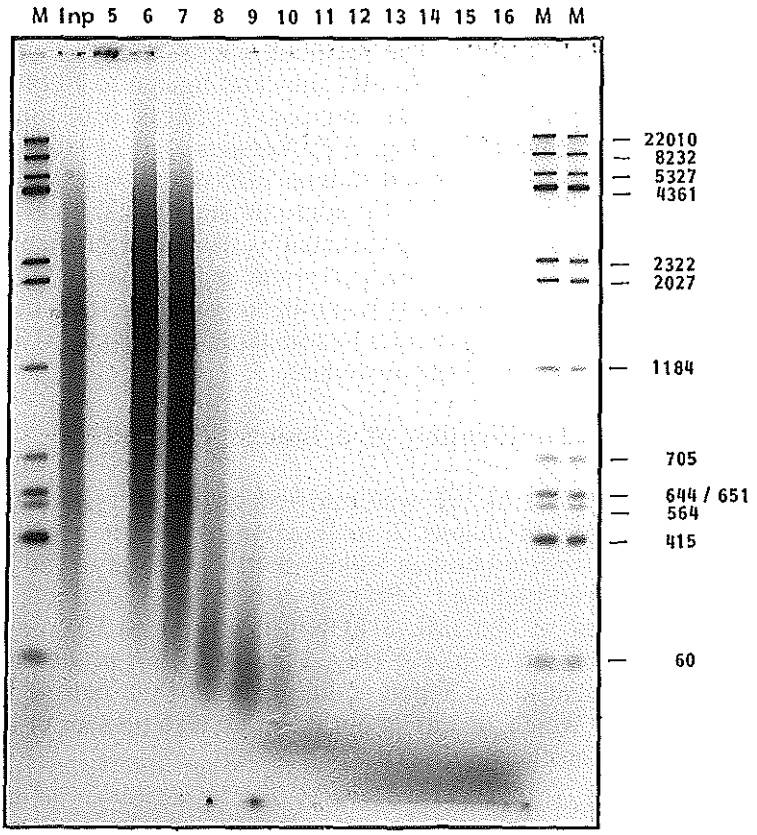
M Inp 5 6 7 8 9 10 11 12 13 14 15 16 M M
22010
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5327
4361
2322
2027
1184
705
644 / 651
564
415
60

Fig. 3.2 Analysis of cDNA of human PBLs-derived mRNA. Double stranded cDNA was constructed of mRNA isolated from human lymphocytes which were stimulated with Concanavalin A and TPA. The cDNA was radiolabeled during second strand synthesis and analyzed on a 1% agarose gel (bottom panel: lane inp). Following ligation of *Eco*RI linkers and digestion with *Eco*RI, the cDNA was fractionated on a 25 x 0.3 cm Sepharose CL-4B column (top panel) and fractions 5-16 were analyzed by agarose gel electrophoresis (bottom panel, lanes 5-16). End labeled lambda DNA digested with *Hind*III and *Bgl*II was used as size marker (lane M).

Since the coding region of a gene is subject to functional constraints, it is assumed that this part of a gene is generally conserved best. Based on this assumption, 1.5 x 10^5 plaques of the human lymphocyte cDNA library were screened, initially, with the radiolabeled SP6-transcribed RNA probe lacking the 3'-untranslated region. No hybridizing clones were identified. Therefore, we finally screened the hIL-3 cDNA library with the complete mIL-3 cDNA which resulted in the identification of four hIL-3 cDNA clones (Fig. 3.3). Positive clones were rescreened and plaque purified. The *Eco*RI cDNA inserts were purified on a 5% polyacrylamide gel and subcloned into M13mp18 or pTZ18R (Pharmacia) DNA.

Restriction enzyme analysis, southern blot hybridization experiments and sequence determination of the isolated clones D11 (910 bp), II (± 400 bp), VI (279 bp) and IV (204 bp) revealed that the smaller clones were identical to the 3'-terminal sequence of the largest clone (D11). Restriction enzyme analysis of clone D11 indicated an internal *Eco*RI site (Fig 3.4; nt position 411 [Fig 3.5). Southern blot analysis of human genomic DNA digested with *Hind*III or *Bam*HI revealed that fragments of 15 kb and 4.6 kb were found to hybridize to probes made from both the 5' and 3' *Eco*RI fragments of clone D11 (not shown).

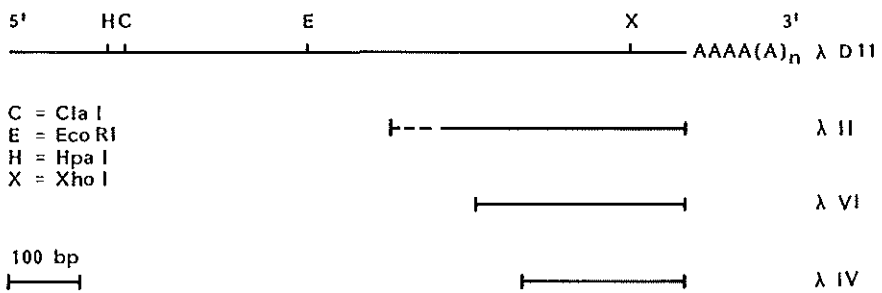

Fig. 3.3 Schematic representation of the hIL-3 cDNA inserts present in lambda gt10 phage DNA. Clones (D11, II, IV and VI) hybridizing with the complete mIL-3 probe are shown. The position of relevant restriction endonuclease sites are indicated.

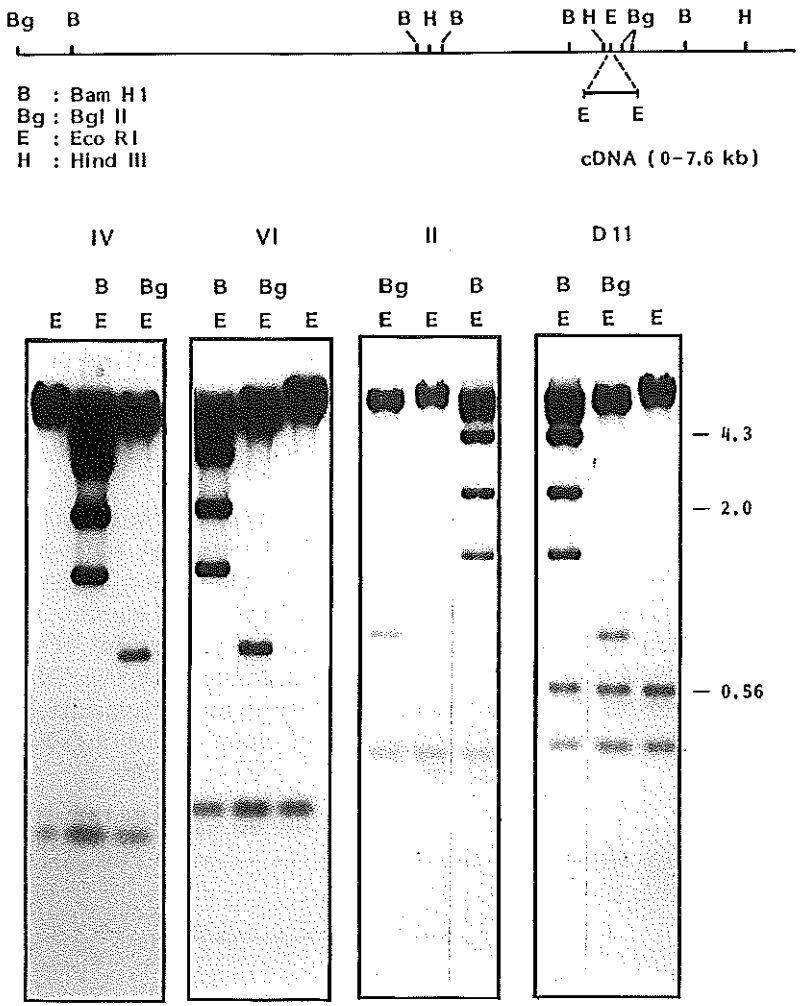

Fig. 3.4 Southern blot analysis of phage DNA isolated from clones hybridizing with mIL-3. Top panel shows restriction map lambda gt10 vector DNA. The *Eco*RI insertion site for the cDNA is indicated. The bottom panel shows southern blot hybridization of the isolated clones. The mIL-3 positive phage DNA was digested with *Eco*R1 and *Bam*H1 or *Bgl*II as indicated, separated by agarose gel electrophoresis and transferred to nylon membrane (Gene Screen Plus, Amersham). The blots were hybridized with the complete D11 phage DNA. Inserts of clones II, IV and VI are homologous to the insert of clone D11. The high molecular weight bands (above 0.6 kb) represent cross-hybridization of vector (lambda phage) DNA.

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                                                    MetSerArgLeuProValLeu
  1 .GACCAGAACAAGACAGAGTGCCTCCTGCCGATCCAAACATGAGCCGCCTGCCCGTCCTG   59

                                                    ▼
    LeuLeuLeuGlnLeuLeuValArgProGlyLeuGlnAlaProMetThrGlnThrThrPro
 60 CTCCTGCTCCAACTCCTGGTCCGCCCCGGACTCCAAGCTCCCATGACCCAGACAACGCCC   119

    LeuLysThrSerTrpValAsnCysSerAsnMetIleAspGluIleIleThrHisLeuLys
120 TTGAAGACAAGCTGGGTTAACTGCTCTAACATGATCGATGAAATTATAACACACTTAAAG   179

    GlnProProLeuProLeuLeuAspPheAsnAsnLeuAsnGlyGluAspGlnAspIleLeu
180 CAGCCACCTTTGCCTTTGCTGGACTTCAACAACCTCAATGGGGAAGACCAAGACATTCTG   239

    MetGluAsnAsnLeuArgArgProAsnLeuGluAlaPheAsnArgAlaValLysSerLeu
240 ATGGAAAATAACCTTCGAAGGCCAAACCTGGAGGCATTCAACAGGGCTGTCAAGAGTTTA   299

    GlnAsnAlaSerAlaIleGluSerIleLeuLysAsnLeuLeuProCysLeuProLeuAla
300 CAGAACGCATCAGCAATTGAGAGCATTCTTAAAAATCTCCTGCCATGTCTGCCCCTGGCC   359

    ThrAlaAlaProThrArgHisProIleHisIleLysAspGlyAspTrpAsnGluPheArg
360 ACGGCCGCACCCACGCGACATCCAATCCATATCAAGGACGGTGACTGGAATGAATTCCGG   419

    ArgLysLeuThrPheTyrLeuLysThrLeuGluAsnAlaGlnAlaGlnGlnThrThrLeu
420 AGGAAACTGACGTTCTATCTGAAAACCCTTGAGAATGCGCAGGCTCAACAGACGACTTTG   479

    SerLeuAlaIlePhe***
480 AGCCTCGCGATCTTTTGAGTCCAACGTCCAGCTCGTTCTCTGGGCCTTCTCACCACAGAG   539

540 CCTCGGGACATCAAAAACAGCAGAACTTCTGAAACCTCTGGGTCATCTCTCACACATTCC   599

600 AGGACCAGAAGCATTTCACCTTTTCCTGCGGCATCAGATGAATTGTTAATTATCTAATTT   659

660 CTGAAATGTGCAGCTCCCATTTGGCCTTGTGCGGTTGTGTTCTCATTTTTATCCCATTGA   719

720 GACTATTTATTTATGTATGTATGTATTTATTTATTTATTGCCTGGAGTGTGAACTGTATT   779

780 TATTTTAGCAGAGGAGCCATGTCCTGCTGCTTCTGCAAAAAACTCAGAGTGGGGTGGGGA   839

840 GCATGTTCATTTGTACCTCGAGTTTTAAACTGGTTCCTAGGGATGTGTGAGAATAAACTA   899

900 GACTCTGAACA  910
```

Fig. 3.5 Human IL-3 nucleotide and deduced protein sequence of the cDNA insert of clone D11. The putative cleavage site for the leader peptide (▽) and potential glycosylation sites (overlined) are indicated.

Furthermore, the nucleotide sequence around the *Eco*RI site did not correspond to the linker sequence (pCCGAATTCGG) used for inserting cDNA into λgt10 phage DNA. These results indicated that the *Eco*RI site was a naturally occurring site and was not the result of the ligation of two independent cDNA fragments. The nucleotide sequences of the various hIL-3 cDNA clones were established as described in chapter 2 and the sequence of the complete hIL-3 cDNA (clone D11) is shown in Fig 3.5.

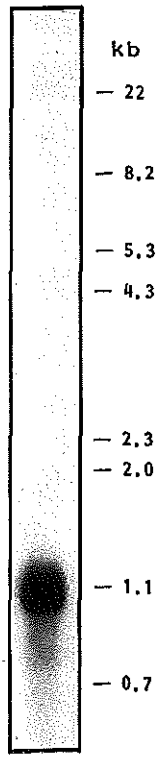

Fig. 3.6 Northern analysis of human PBLs for IL-3 transcripts. Northern blot containing poly (A)+ RNA isolated from activated human PBLs was hybridized with a hIL-3 cDNA probe. The RNA sample used for northern analysis was the same to that used for cDNA synthesis. A single IL-3 transcript of approximately 1.1 kb was identified. Molecular sizes are indicated on the right (in kilobases).

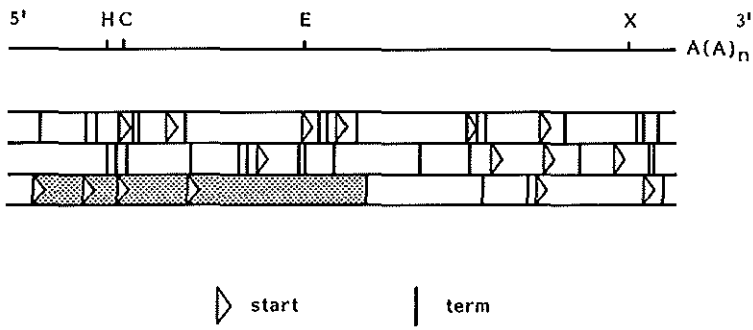

Fig. 3.7 Translation of the human IL-3 cDNA (D11) in three possible open

reading frames. Translation initiation and termination codons are indicated. Dashed box shows the largest and actual open reading frame.

Analysis of expression of mRNA in human peripheral blood lymphocytes (PBLs) following stimulation with ConA and TPA by standard RNA blotting analysis (Northern blots) using the insert of clone D11 revealed that the naturally occurring human IL-3 transcript is approximately 1.1 kb in length (Fig. 3.6). Assuming that the isolated cDNA clone D11 is a full-length cDNA comprising all the coding regions, the natural transcript in PBLs is apparently polyadenylated with approximately 200 A residues, which is in the expected range. Furthermore, it was estimated from these Northern blots as well as from the incidence of the IL-2 transcripts in the constructed phage cDNA library that the amount of IL-3 transcripts is at least 10-fold lower than that of IL-2. Furthermore, it was estimated that the content of IL-3 mRNA present in the total amount of mRNA in stimulated PBLs ranged from 0.01% - 0.001%.

The isolated hIL-3 cDNA contains an open reading frame (Fig. 3.7; dashed box) from the putative start codon at position 39-41 up to the termination codon TGA at position 495-497 (Fig 3.5). The ATG triplet at position 39-41, resulting in the largest open reading frame, was postulated as the start codon. Utilization of this first ATG as the actual start codon is further supported by the presence of an A residue at position -3 relative to this ATG triplet which was shown to have a dominant effect on the efficiency of the start codon (Kozak, 1986). The putative encoded protein is probably processed at the amino terminus by cleaving the precursor polypeptide between the glycine and the alanine residues as could be predicted from a recent method to identify signal sequence cleavage sites (Von Heijne, 1986). The predicted precursor polypeptide, comprising 152 amino acids (aa), consists of a hydrophobic leader peptide of 19 residues and a mature protein of 133 aa (M_r 15,091).

3.5 Sequence comparison between human and murine IL-3 cDNA

Computer-assisted alignment (Fig. 3.8) of the hIL-3 cDNA (clone D11) and the mIL-3 cDNA (Fung et al., 1984; Miyatake et al., 1985) revealed significant DNA homology in the first 100 bp and between nt 598-803 (68%, and 73%, respectively). In particular, the 3'-terminal region between nt 706 and 763 is highly conserved and displays a homology of 93%. This significant homology in the 3'-untranslated region is clearly visible on the computer-assisted matrix construction of the human and murine IL-3 cDNA (Fig. 3.9). Analysis of this highly conserved domain in the 3'-terminal non-coding region, reveals the presence of eight ATTTA repeat units in the murine sequence and six in the human sequence.

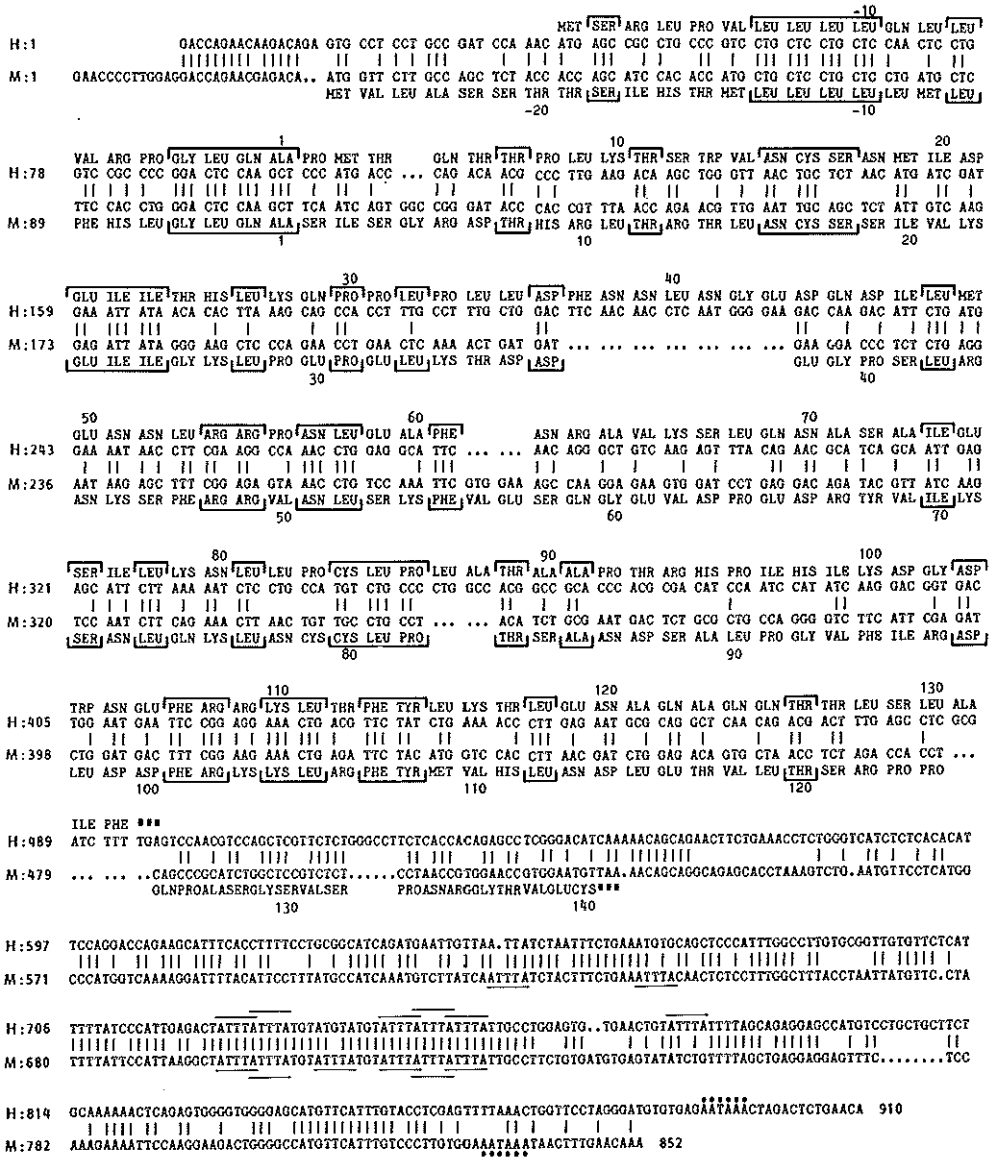

Fig. 3.8 Comparison of nucleotide and amino acid sequences of human IL-3 (H) and mouse IL-3 (M). The human IL-3 protein and nucleotide sequence (clone D11, top lines)

were aligned with those for the mIL-3 DNA (Fung et al., 1984; Miyatake et al., 1985) and protein (Clark-Lewis et al., 1986. Numbers at the left refer to the first nucleotide in the corresponding line. Amino acid numbers are presented above or below the appropriate amino acid residue. Identical nucleotides are connected by vertical lines, identical amino acids are enclosed in horizontal brackets. Black dots in the bottom lines indicate polyadenylation signal sequences and horizontal bars marks ATTTA repeat units. Sequence alignments were performed using various computer programs (Staden, 1982; Devereux et al., 1984; Queen and Korn, 1984; Lipman and Pearson, 1985) and by visual inspection.

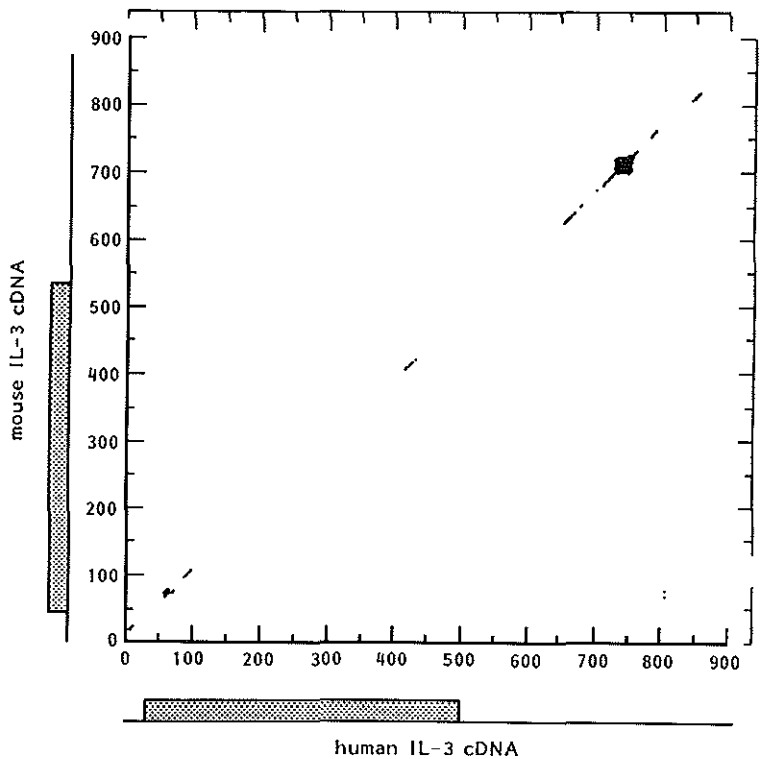

Fig. 3.9 Computer-assisted matrix plot of homology between human IL-3 and mouse IL-3. The matrix plot was constructed using the Diagon program in the Staden package. The complete human IL-3 cDNA (x axis) was plotted against the complete mouse IL-3 cDNA (y axis). The dotted boxes represent the IL-3 coding regions, whereas the 5' and 3' noncoding regions of the IL-3 cDNA are indicated as solid lines. Each point in the plot represents 12 identities in a stretch of 15 nucleotides.

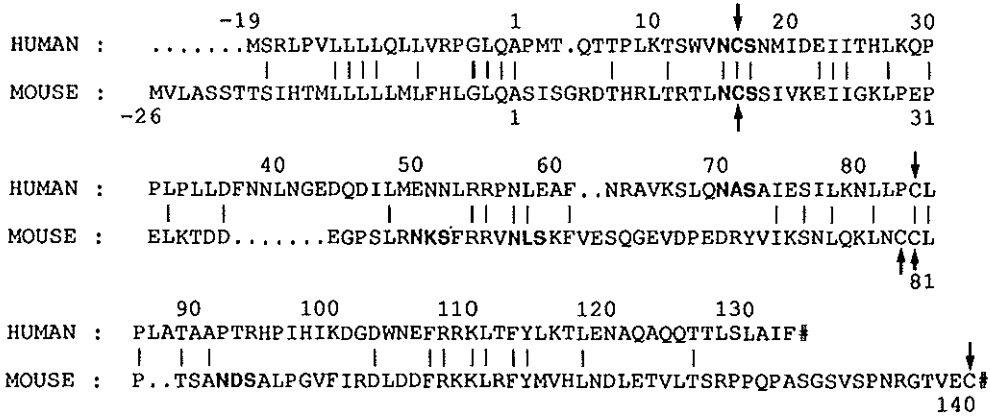

Fig. 3.10 Alignment of the protein sequences of human and mouse IL-3. For details on the alignment and numbering see Fig. 3.8. The protein sequences are shown in the single-letter code and aa identities between human and mouse IL-3 protein are connected by vertical lines. Numbering is relative to the first aa of the mature IL-3 protein. Gaps introduced to maximize the homology are represented by dots. Cysteine residues are indicated by arrows and potential glycosylation sites are marked as well (bold).

With respect to their position in the cDNA, five repeat units present in the human sequence appeared to be preserved in the murine sequence as well (Fig 3.8). The low homology in the 5'-terminal 600 bp of the human cDNA (52%) precludes detection by hybridization with the *Hind*III-*Xba*I fragment of mIL-3 under the stringent conditions used. The coding region of hIL-3 shares only 45% homology with the corresponding murine sequence.

The alignment of the predicted aa sequence encoded by the D11 cDNA clone and mouse IL-3 (Fig. 3.10) reveals a homology of 50% for the leader peptide (aa -19 to +1) and only 28% for the mature protein (aa 1 to 133). The region which encloses the processing site (residues -3 to +1) as well as the region from residue -13 to -10, both present in the leader peptide, have been completely conserved in these species. The mature protein has four reasonably conserved domains (aa 15-36, 54-61, 74-91 and 107-118) and contains two potential N-linked glycosylation sites (pos. 15-17 and 70-72). The two cysteine residues present in the mature protein at position 16 and 84 are conserved and may play an essential role in protein folding by disulfide bridge formation.

Table 3.1 Segregation of hIL-3 with human chromosomes in human-rodent somatic hybrids[a]

Human chromosome	Number of hybrids with indicated chromosome/hIL-3 fragment pattern				
	concordant hybrids		discordant hybrids		total discordant hybrids
	(+ / +)	(- / -)	(- / +)	(+ / -)	(% discordance)
1	7	4	2	7	9 (45)
2	4	9	5	2	7 (35)
3	3	5	5	5	10 (56)[b]
4	4	8	5	3	8 (40)
5	**9**	**10**	**0**	**1**	**1 (5)**
6	6	2	3	9	12 (60)
7	6	4	3	7	11 (55)
8	6	1	3	10	13 (65)
9	3	7	6	4	10 (50)
10	3	3	6	8	14 (70)
11	3	7	2	3	5 (33)[b]
12	4	5	5	6	11 (55)
13	5	5	4	6	10 (50)
14	5	7	4	4	8 (40)
15	5	7	4	4	8 (40)
16	6	8	3	3	6 (30)
17	3	3	6	7	13 (68)[b]
18	5	8	4	3	7 (35)
19	3	5	6	6	12 (60)
20	8	3	1	8	9 (45)
21	4	4	5	7	12 (60)
22	6	2	3	6	9 (53)[b]
X	5	1	3	10	13 (68)[b]

[a] DNA of 20 somatic cell hybrids of human and rodent cells were examined for the presence of hIL-3 sequences by Southern blot analysis as described in chapter 2. Data are presented per chromosome, whereby +/. and -/. indicate the number of hybrids with that particular human chromosome present or absent, respectively. The presence or absence of specific hIL-3 hybridization is referred to as ./+ and ./-, respectively. For example, 14 hybrids have retained chromosome 1 of which only 7 show hIL-3 specific hybridization (+/+) and 7 do not (+/-). Hybrids without chromosome 1 (n=6) contain hIL-3 specific DNA sequences in only two cases (-/+). The number of discordant hybrids and the corresponding percent discordance are shown. The low percent discordance (e.g. 0% indicates a matched segregation of the probe with a chromosome) of the hIL-3 probe with the underlined and bold marked somatic hybrid was the basis for the assignment of IL-3 to human chromosome 5

[b] Cell hybrids containing specific chromosome fragments or containing chromosome rearrangements were omitted from the analysis of that particular chromosome.

3.6 Chromosomal mapping of human IL-3

The chromosomal localization of the gene encoding hIL-3 was established by southern blot analysis of a panel of somatic cell hybrids between human and rodent cells (Geurts van Kessel et al., 1983). DNA isolated from the somatic cell hybrids and control DNA of human leukocytes and rodent cells was digested with *Bam*HI or *Eco*RI, size-fractionated by agarose gel electrophoresis, transferred onto nylon membranes and hybridized to a radiolabeled hIL-3 cDNA fragment (nt 1-541). Since this human probe does not hybridize to rodent DNA under the stringency conditions used, the hybridizing restriction fragments were exclusively indicative for the presence of the human gene. Table 3.1 shows the correlation between human chromosomes and restriction fragments hybridizing to the hIL-3 cDNA probe. The data clearly indicate that hIL-3 homologous sequences segregated with human chromosome 5 in the cell hybrids studied. The single observed discordance may be due to a cytogenetically undetected small deletion in chromosome 5, which may have occurred in this particular cell line after cell fusion. Discordances with other chromosomes were significantly higher, indicating that the hIL-3 gene is located on chromosome 5.

3.7 Expression of human IL-3 in COS cells

Expression of genes in COS cells is generally faster and less complicated than in other expression systems. In order to verify whether the isolated human cDNA encodes a functional protein that biologically resembles mIL-3, the D11 cDNA was inserted in an eukaryotic expression vector pLO (for detailed description see chapter 2), containing a SV40 transcription unit. The resulting pLB4 (Fig. 3.11) plasmid was isolated following propagation in *E. coli.* and closed circular plasmid DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients as described in chapter 2. Highly purified pLB4 plasmid DNA was transfected to COS-1 cells to express the encoded protein of the inserted cDNA.

COS cell supernatant was harvested 48-72 hours after transfection and subsequently screened for the presence of biologically active hIL-3 by testing for its capacity to stimulate colony formation of normal human bone marrow as well as its capacity to induce proliferation of human acute myelogenous leukemia (AML) blasts. The biological data presented in chapter 5 demonstrate that the isolated D11 cDNA clone contains the genetic information for a biologically active protein which is transiently expressed and excreted into the culture medium by COS-1 cells. Furthermore, conditioned medium of hIL-3 transfected COS cells appeared to contain approximately 10 to 100 units per ml of supernatant (see also chapter 5 for biological activity and section 2.2.2 for IL-3 unit definition), which corresponds with 0.01 µg to 0.1 µg per ml. The

high consumption of purified plasmid DNA due to the transient expression of the transfected gene and the relatively low expression level are the major disadvantages of the COS expression system. Therefore, we set out for other systems, including both eukaryotic and prokaryotic systems, to express the isolated hIL-3 cDNA.

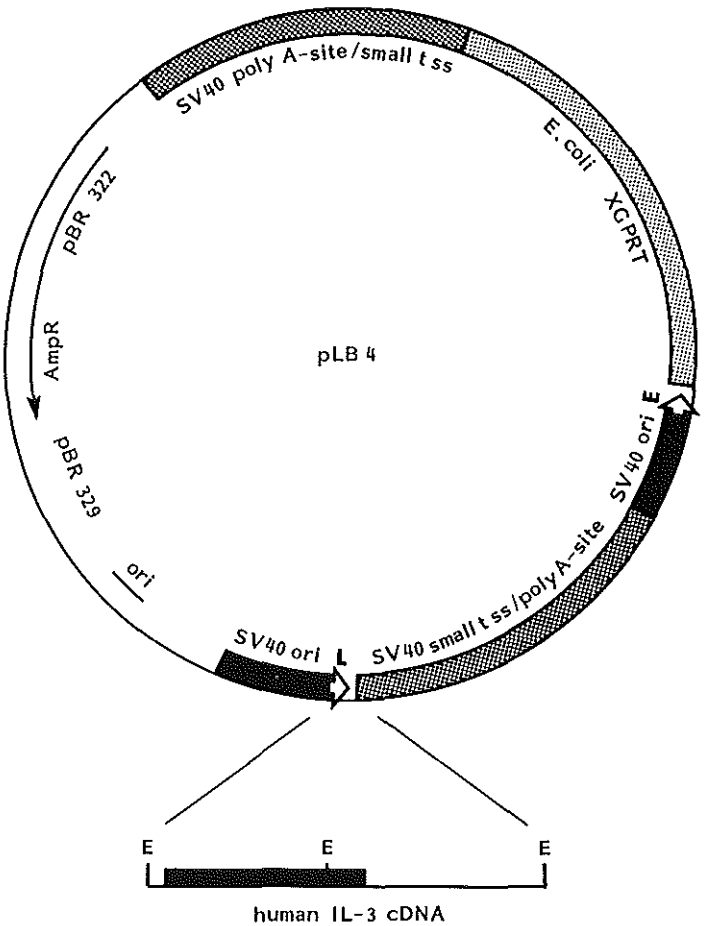

Fig. 3.11 Eukaryotic expression vector containing hIL-3 cDNA. The pLB4 plasmid was constructed by inserting hIL-3 cDNA, isolated from clone D11 by partial EcoRI digestion, into the unique *Eco*RI site of pL0 (see chapter 2 section 2.1.1). Plasmid pLB4 contains an complete SV40 transcription unit, i.e., both SV40 origins of replication (early and late promoter) and two small t-antigen splice and polyadenylation sequences. The bacterial origin of replication (ori pBR 329) and the ampicillin resistance gene (AmpR pBR 322) and the E. coli XGPRT gene are indicated as well. The human IL-3 cDNA was inserted downstream of the SV40 late promoter.

3.8 Production and purification of *E. coli* synthesized lacZ/hIL-3 fusion proteins and production of antibodies directed against human IL-3

The preliminary characterization of recombinant hIL-3 was achieved by expression of the isolated D11 cDNA in COS-1 cells. However, the production of hIL-3 following transfection of an eukaryotic expression vector to COS-1 cells is generally rather low. Various bacterial expression vectors for the production of fusion proteins between hIL-3 and a part of the lacZ protein were constructed (see chapter 2). The plasmids, i.e., pUC/hMulti, pUC/hMultiΔ1 and pUC/hMultiΔ2 contain the hIL-3 coding sequences which are placed downstream of the translation initiation site of the lac operon resulting in the expression of fusion proteins (Fig. 3.12).

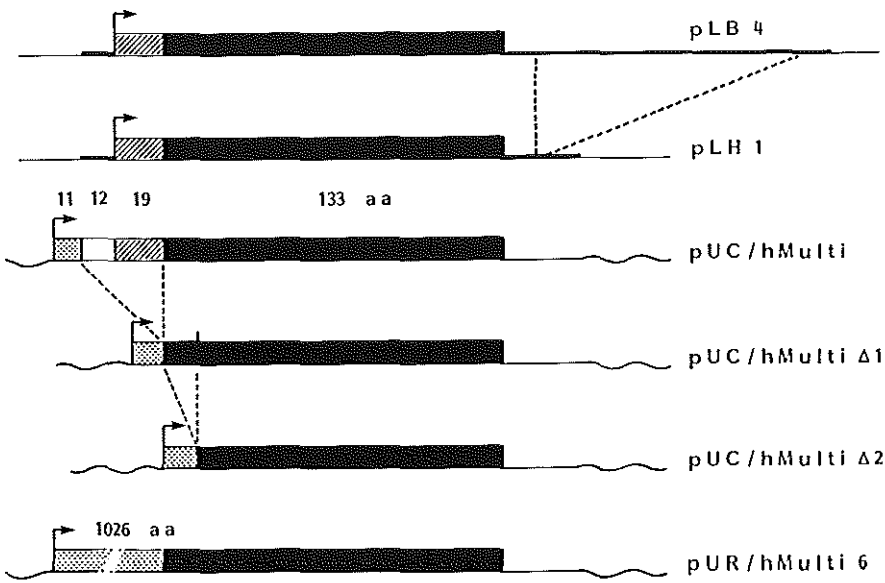

Fig. 3.12 Schematic representation of human IL-3 expression vectors. The IL-3 cDNA inserts of the eukaryotic expression vectors pLB4 and pLH1 are shown. Plasmid pLB4 contains the complete hIL-3 cDNA, whereas in pLH1 plasmid most of the 3' untranslated sequences are deleted. The leader peptide and the mature IL-3 protein region are indicated by dashed and black boxes, respectively. The bacterial expression vectors (pUC/hMulti, pUC/hMultiΔ1, pUC/hMultiΔ2, and pUR/hMulti 6) contain, as indicated, lacZ derived sequences (dotted box), 5' untranslated sequences of hIL-3 (open box), the hIL-3 leader peptide (dashed box) and the hIL-3 mature protein (black box). The pUC/hMultiΔ2 encodes a protein of 11 aa of lacZ fused to aa 15-133 of the mature hIL-3 protein. The number of aa derived from particular sequences are indicated. The arrow marks the ATG start codon used in the particular vector. See also chapter 2 for detailed description of these expression vectors.

The plasmid pUC/hMulti encodes a fusion protein composed of the first 11 aa of β-galactosidase, 12 aa noncoding 5' hIL-3, 19 aa hIL-3 signal peptide and 133 aa mature hIL-3 (Fig. 3.12). Thus, the pUC/hMulti encodes a 20 kDa fusion protein which includes the hydrophobic signal peptide of hIL-3 (Fig. 3.12).The plasmid pUC/hMultiΔ1 was constructed to produce a fusion protein in which the 5' noncoding part as well as the signal peptide are deleted. This vector encodes a 16 kDa fusion protein comprising the first 11 aa of lacZ fused to aa 2 to 133 of the mature hIL-3 polypeptide (Fig. 3.12). The pUC/hMultiΔ2 vector, a side-product obtained during the construction of pUC/hMultiΔ1 (see chapter 2), encodes a hIL-3 fusion protein with a molecular weight of approximately 15 kDa which consists of the first 11 aa of lacZ fused to aa 15 to 133 of the mature hIL-3 polypeptide (Fig. 3.12).

After introducing these bacterial expression vectors into *E. coli* (JM109) the production of fusion proteins was initiated/enhanced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to the culture medium as an inducer of the transcription of the lac operon. It appeared that most of the produced hIL-3 fusion protein was stored in the bacteria in an insoluble form which enabled the use of a simple and efficient purification procedure (see chapter 2). Disruption of the bacteria through sonication followed by extraction with nonionic detergents such as NP-40 (see 2.3.2) resulted in the solubilization of most of the bacterial proteins (Fig. 3.13, lanes 1 and 2). The hIL-3 fusion proteins, stored in inclusion bodies of the bacteria and insoluble under these conditions, were solubilized by re-extracting the remaining fraction with a high concentration (8M) urea buffer. Fig. 3.13 lane 3 shows that a highly effective purification of the hIL-3 fusion proteins was achieved with this rather simple purification protocol.

The high production level of the hIL-3 fusion proteins, together with the availability of an efficient purification protocol, enabled the production of antibodies directed against the hIL-3 polypeptide. The 20 kDa fusion protein was purified as described in chapter 2 and finally subjected to preparative SDS polyacrylamide gel electrophoresis. The band representing hIL-3 with only minor contaminants was excised from the gel and the ensuing gel slice was pulverized prior to the immunization of a rabbit (polyclonal antiserum) and Balb/C mice (monoclonal mouse anti-human IL-3 antiserum) as described elsewhere (Dorssers et al., 1987b).The resulting polyclonal antiserum reacted with the bacterially produced hIL-3 fusion proteins, e.g. hIL-3 fusion proteins produced by pUC/hMulti and pUC/hMultiΔ1 (Fig. 3.14 lanes 1 and 2, respectively). To confirm the specificity of the polyclonal antiserum, a 130 kDa hIL-3 fusion protein was produced. This large fusion protein was encoded by the pUR/hMulti 6 vector (Fig. 3.12) in which the mature hIL-3 sequences are fused to the 3'-terminal part of the β-galactosidase gene in the pUR 288 plasmid (Rüther and Müller-Hill, 1983).

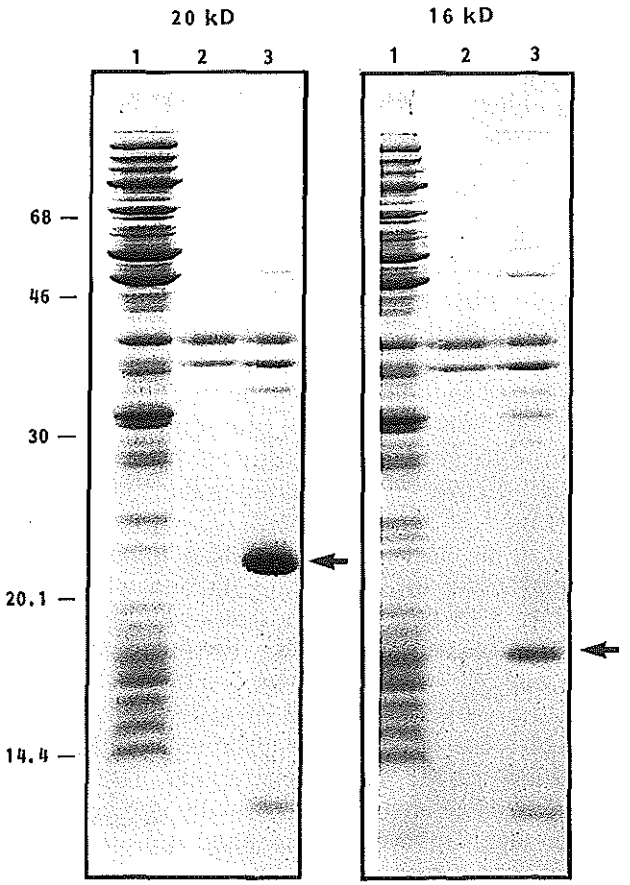

Fig. 3.13 Coomassie Brilliant Blue stained SDS-PAGE of hIL-3 fusion proteins. Left panel shows lacZ/hIL-3 fusion proteins (20 kDa) purified from bacteria transformed with pUC/hMulti. Right panel shows 16 kDa hIL-3 fusion proteins derived from pUC/hMultiΔ1. Bacterial proteins extracted with 0.2% NP-40 (lanes 1, samples correspond to 0.1 ml of the original bacterial culture), bacterial proteins reextracted with 0.5% NP-40 (lanes 2, 0.2 ml original culture), and solubilized hIL-3 fusion proteins by sonication in 8 M urea (lanes 3, 0.2 ml of original culture) were separated on a 13.5 % SDS polyacrylamide gel. Human IL-3 fusion proteins are indicated with arrows. Molecular weights (kDa) of marker proteins (lane M) are denoted on the left.

The 130 kDa fusion protein also reacted with the polyclonal antiserum and the 117 kDa β-galactosidase which was produced by bacteria transfected with pUR 288 did not react with the antiserum (Fig. 3.14 lanes 4 and 3, respectively). These results demonstrate that the polyclonal antiserum is capable of an immunospecific reaction with hIL-3. The polyclonal as well as the monoclonal antisera have been extensively characterized using hIL-3 mutants which resulted in the identification of several monoclonal antibodies directed against different antigenic epitopes present on the hIL-3 polypeptide (Dorssers et al., 1991).

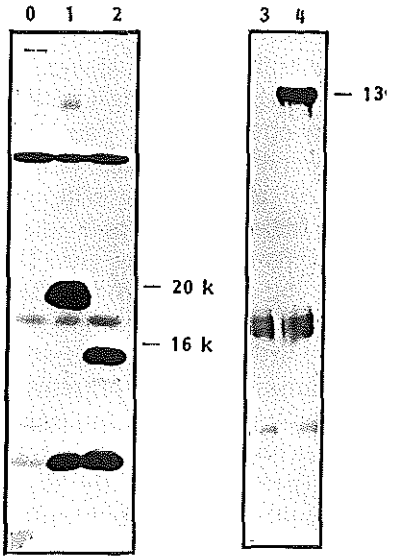

Fig. 3.14 Immunological analysis of hIL-3 fusion proteins. Bacterial proteins (lane 0: pUC19; lane 1: pUC/hMulti; lane 3: pUC/hMultiΔ1; lane 4: pUR 288; lane 5: pUR/hMulti 6) were separated on 13.5% (left panel) or 7.5% (right panel) SDS polyacrylamide gels and transferred onto nitrocellulose membranes using the semi-dry transfer technique. Upon extraction with 0.5% NP-40, the 16 and 20 Kda hIL-3 fusion proteins were solubilized by sonication in urea buffer (1M urea; 1% NP-40). The 120 kDa hIL-3 fusion protein was applied onto gel as a total cell lysate. Filters were incubated with polyclonal rabbit anti-hIL-3 (1:250 diluted) as described in chapter 2. Immune complexes were visualized by anti-rabbit IgG, streptavidin-horseradish peroxidase conjugates and 4-chloro-1-naphthol as substrate. Details of the western blot analysis are described in section 2.3.1.

The biological activity of these fusion proteins was determined, initially, in the AML proliferation assay (Delwel et al., 1987; chapter 2 and 5). The 15 and 16 kDa hIL-3 fusion proteins exerted half-maximal stimulation of proliferation of AML blasts (see chapter 5) at a concentration of about 1 ng/ml. The amount of hIL-3 fusion protein as well as the total protein content were estimated from a coomassie brilliant blue stained SDS polyacrylamide gel. The 20 kDa hIL-3 fusion protein appeared to be approximately 100-fold less active than the 15 and 16 kDa proteins. This observation can be explained by the presence of the hydrophobic leader peptide in the 20 kDa protein which most likely caused

conformational changes leading to reduced biological activity. Furthermore, the 130 kDa hIL-3 fusion protein did not exert any detectable biological activity on the AML blast cells.

3.9 Large-scale production and subsequent purification of the hIL-3 protein

To facilitate large scale production of hIL-3 by expression of the isolated hIL-3 cDNA, a number of expression vectors were constructed and transfected to appropriate host strains, i.e., *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Bacillus licheniformis* (*B. licheniformis*), *Kluyveromyces lactis* (*K. lactis*), *Saccharomyces cerevisiae* (*S. cerevisiae*) and C127 murine cells (Dorssers et al., 1987b; Van Leen et al., 1990; Van Leen et al., 1991).

In order to establish stable vertebrate cell lines capable of producing hIL-3 in a more natural form, i.e., carrying the complex type of glycosylation characteristic for higher eukaryotes, C127 and CHO cells were transfected with a pLB4 derived plasmid in which the entire bovine papillomavirus-1 (BPV-1) DNA was inserted as described elsewhere (Dorssers et al., 1987b; Van Leen et al., 1990). The general idea of using the BPV-1 genome is that this virus can readily transform a variety of rodent cells *in vitro* and is very efficient in inducing transformed foci which are picked from the culture dishes. Transformed cells contain multiple copies (10 to 120 per cell) of the viral DNA, as unintegrated but stably maintained molecules. The genetics of the BPV-1 genome is rather complicated due to the presence of multiple promoters and both transcriptional enhancers and repressors. Furthermore, differential splicing of RNA results in distinct open reading frames encoding proteins that have different functions, including transactivating activities, DNA binding and dimer formation activities and transcriptional repressing activities. Therefore, transcription of homologous and heterologous genes driven by distinct promoters in this system will be under tight control of these regulatory elements. Improved productivity according to alterations in the BPV-1 expression system is described elsewhere (Persoon and Van Leen, 1990). Supernatant of hIL-3/BPV transfected cells was tested for IL-3 activity (chapter 5) using either the AML proliferation system or the IL-3 dependent human AML-193 cells.

To determine which expression system is the most efficient for large-scale production of hIL-3, the hIL-3 gene was introduced and expressed in bacteria, including *E. coli* and *Bacillus* (*B.*) species such as *B. subtilis* and *B. licheniformis*, but also in yeast, in particular *Kluyveromyces lactis* (*K. lactis*) and *Saccharomyces cerevisiae* (*S. cerevisiae*). Construction of suitable expression vectors and introduction in the different microorganisms as well as analysis of the produced proteins is described elsewhere (Dorssers et al., 1987b; Persoon and Van Leen, 1990; Van Leen et al., 1991). The recombinant hIL-3

preparations derived from these different microorganisms or host strains were tested for their capacity to stimulate hemopoietic precursor cells. All recombinant hIL-3 proteins appeared to be biologically active. A selection of the biological data will be presented and discussed in chapter 5. Furthermore, these studies demonstrated that the *Bacillus* species, especially *B. licheniformis*, were the eligible candidates to obtain large-scale production of well-defined recombinant hIL-3. Following several improvements of the Bacillus expression vector (e.g. exchange of the *Hpa*II promoter by the strong α-amylase promoter), the resulting optimal vector contains downstream of the α-amylase promoter coding information for the *Bacillus* α-amylase signal perfectly fused to the sequences encoding mature hIL-3. Furthermore, the α-amylase signal/mature hIL-3 open reading frame (ORF) was located upstream of the 3' end of the amylase gene containing the amylase terminator (Fig. 3.15; Persoon and Van Leen, 1990; Van Leen et al., 1990; Van Leen et al., 1991). Finally, the resulting plasmid was transformed to *B. licheniformis* strain T9 to synthesize recombinant hIL-3. The major advantages of this production system are high expression level (>5 mg/l) and secretion of more than 95% of the synthesized mature protein into the culture medium.

The latter property of this expression system enabled the use of a relatively simple and highly efficient purification scheme for recombinant hIL-3 produced by *Bacillus* species. The *Bacillus*-derived unglycosylated recombinant hIL-3 was purified to homogeneity by a series of chromatographic steps including hydrophobic interaction and anion-exchange chromatography and mostly followed by gel filtration to separate low molecular weight proteins. The different chromatographic purification procedures are described in chapter 2 and the results of this three-step purification protocol will be briefly described in the next paragraph.

In the initial step, hIL-3 present in the cell free medium from *B. licheniformis* T9 was adsorbed to a hydrophobic matrix whereas most of the protein was found in the run-through fractions. Bound proteins were eluted with a steep ammonium sulfate [$(NH_4)_2SO_4$] gradient and the enriched hIL-3 fractions were concentrated by $(NH_4)_2SO_4$ precipitation at 70% saturation.

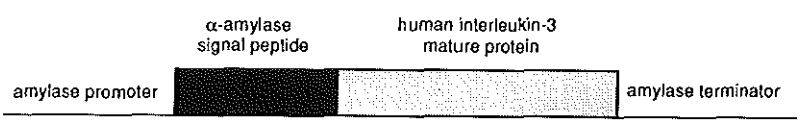

Fig. 3.15 Schematic representation of the hIL-3 expression cassette used for large-scale production in *Bacillus licheniformis*.

The precipitate, dissolved and desalted, was subjected to anion-exchange chromatography. Whereas the hIL-3 activity was found in the run-through fractions, most of the contaminating proteins appeared to be bound to the column. The hIL-3 enriched run-through fractions were concentrated and subsequently separated from low molecular weight contaminants, if any, by gelfiltration. This purification protocol was shown to be highly effective, resulting in hIL-3 preparations that are free from detectable non-hIL-3-like protein , as was demonstrated by Coomassie brilliant blue stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis using antibodies directed against the hIL-3 polypeptide (Fig. 3.16). N-terminal sequencing of the *Bacillus*-derived homogenous hIL-3 showed that the synthesized protein has the correct amino terminal sequence. Furthermore, proteolytic degradation products occasionally present in the culture medium obtained from *B. licheniformis*T9 (T9 is an exoprotease negative and endoprotease positive strain) were selectively removed using various purification procedures as described elsewhere (Persoon and Van Leen, 1990).

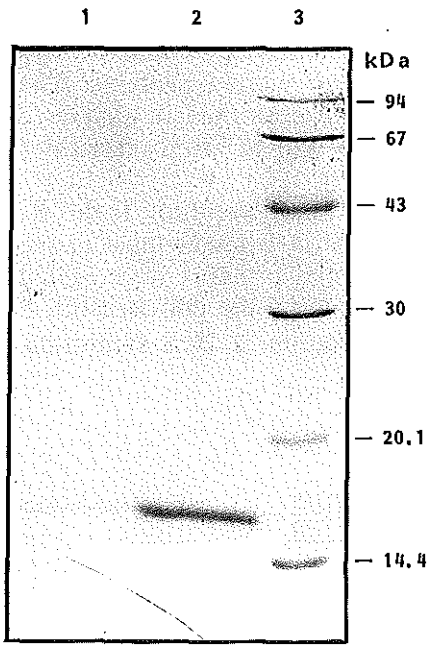

Fig. 3.16 Coomassie Brilliant Blue stained SDS-PAGE of purified *B. licheniformis*-derived hIL-3. Lanes 1 and 2 were loaded respectively with 0.1 µg and 1 µg protein of a purified hIL-3 preparation (homogeneous nonglycosylated hIL-3 expressed in *B. licheniformis*). The single Coomassie brilliant blue stained band demonstrated that the hIL-3 preparation has a purity of ≥95%. Lane 3 was loaded with protein markers (LMW, Pharmacia) and their corresponding molecular weights (kDa) are indicated at the right.

3.10 Summary and Discussion

The identification of hIL-3 cDNA constructed from mRNA of mitogen stimulated human leukocytes was established by virtue of hybridization with the 3'-terminal region of the murine IL-3 cDNA. The 3'-untranslated sequences of a gene are not believed to be subject to functional constraints and are generally thought to be less conserved than the coding sequences. The result obtained with the 3' murine IL-3 probe was unexpected and avoided the laborious procedure of functional expression cloning. The 3'-untranslated region from nt 598 to 803 displays 93% sequence homology with the murine counterpart, whereas the coding regions of the isolated cDNA clone (D11) share only 45% homology. Analysis of the 3' noncoding region revealed that highly conserved ATTTA motifs were mainly responsible for the rather high homology of this region between human and mouse. The presence of highly conserved AT-rich sequences in several cytokine genes, such as all identified IL-3 species, human and murine GM-CSF, human and murine IL-2, human γ-IFN, human G-CSF but not M-CSF suggests a critical role for these sequences in the regulation of production of the hemopoietic growth factors.

Attempts to detect IL-3 homologous sequences in human genomic libraries using a murine IL-3 probe devoid of its 3' untranslated region resulted in nonspecific hybridization signals, faint signals or no signal at all depending on the stringency used. Furthermore, inclusion of the 3' noncoding regions in the murine IL-3 cDNA probe significantly increased the homology and enabled detection of the corresponding human sequences. However, the presence of multiple reiterations of the ATTTA motifs in many lymphokines might interfere with the detection of IL-3 by probe-hybridization screening of human genomic libraries and might result in nonspecific hybridization signals. The aspects as outlined here presumably account for the different results with respect to identification of human IL-3 by probe-hybridization techniques among several investigators.

The repeat units have been shown to mediate selective degradation of several lymphokine mRNAs (Shaw and Kamen, 1986). A cytoplasmic protein has been identified that specifically binds to RNA molecules containing several reiterations of the AUUUA structural element. The binding occurs very rapidly resulting in the formation of a stable RNA/adenosine-uridine binding factor complex and likely precedes degradation events of labile mRNA (Malter, 1989). Mutational analysis indicated that three adjacent AUUUA pentamers are required for binding of a factor that mediates rapid IL-3 mRNA decay (Stoecklin et al., 1994). Translational blockades imposed by AU-rich cytokine derived sequences have also been reported (Kruys et al., 1989).

It was demonstrated that the gene corresponding to the isolated cDNA clone (D11)encoding hIL-3 is located on chromosome 5. In addition to the gene encoding hIL-3, the genes of IL-4, IL-5, IL-9, the p40 subunit of IL-12, IL-13,

GM-CSF, CSF-1, and the gene encoding the B isoform of the platelet-derived growth factor receptor (PDGF-R_B) are all located on a small segment of the 5q arm of human chromosome 5 (see chapter 1: Table 1.2). The genes encoding murine IL-3, GM-CSF, IL-4 and IL-5 have been mapped to mouse chromosome 11 (Lee and Young, 1989), indicating conservation of gene localization. Whether this chromosomal association of these genes is fortuitous or suggests that these genes encoding functionally related hemopoietic factors are part of a superfamily of inflammatory response genes is unknown. This specific localization could also relate to the coordinate expression characteristics of many of these proteins.

Loss of a whole chromosome 5 or deletion of a part of the long arm of this chromosome (del[5q]) is frequently observed in malignant cells of patients with specific myeloid disorders, including myelodysplastic syndrome (MDS) and acute nonlymphocytic leukemia (ANLL). The deletions of chromosome 5 observed in ANLL and in the "5q- syndrome", a primary MDS disorder characterized by refractory anemia with abnormal megakaryocytes, are characterized by variability in the proximal and distal breakpoints. However, a clinical evaluation of patients suffering from these disorders revealed a common and apparently critical region, consisting of bands 5q23 and q31, that was deleted in all patients (Le Beau et al., 1987). The loss of genes within this region may play an important role in the pathogenesis of hematologic disorders associated with a del(5q). The genetic consequence of a deletion of such a HGF allele is as yet a matter of speculation. Such an allelic deletion may result in decreased synthesis of the gene product or may allow expression of the other allele on the homologous chromosome. Moreover, loss of function of both alleles may have occurred through a deletion of one allele and dysfunction of the other as a result of mutation(s). This latter mechanism has been confirmed for the pathogenesis of retinoblastoma (Friend et al., 1986). Whether the del(5q) related disorders are in fact the consequence of the loss of HGF genes is as yet unknown.

The isolated hIL-3 cDNA encodes a precursor polypeptide of 152 aa which is most likely processed at the amino terminus by the removal of a leader peptide of 19 residues. As yet, natural hIL-3 has not been purified and examined to confirm the postulated N-terminal residue of natural mature hIL-3. With regard to murine IL-3 (mIL-3), the presence of mature mIL-3 with variable N-terminal residues, as determined by protein sequencing of natural purified secreted mIL-3, has been reported (Ihle et al., 1989). Protein comparison between mature murine and human IL-3 displays only 28% homology and reveals the conservation of two cysteine residues and the presence of two potential N-linked glycosylation sites. The protein homology found for human and murine IL-3 (28%) is considerably less than displayed by other murine and corresponding human cytokines (see chapter 1) such as IL-11 (90%), CNTF (83%), Epo (81%), SCF (80%), TNF-α (79%), IL-1β (78%), MIP1 (77%), IL-

10 (73%), G-CSF (73%), p40 subunit IL-12 (70%), IL-4 (70%), IL-5 (70%), EGF (70%), IL-1α (65%), IL-2 (63%), p35 subunit IL-12 (60%), IL-7(60%), IL-13 (58%), GM-CSF (56%), IL-9 (56%), and IL-6 (42%). The relatively low conservation of IL-3, also reflected in the absence of cross-reactivity, indicates that the IL-3 gene has diverged in evolution more rapidly than the genes for most of the other HGFs. Results obtained from pairwise comparison of IL-3 coding sequences of various species will be presented in chapter 6. The absence of cross-reactivity, i.e., hIL-3 does not stimulate proliferation of murine CFU-S and neither does it support the growth of IL-3 dependent murine cells with hIL-3, strongly indicates that an additional HGF able to stimulate murine cells has to be present in the conditioned medium of stimulated human PBLs. Partial purification of this postulated HGF demonstrated that the factor is distinct from potential candidates such as IL-6 or HILDA/LIF. Whether this factor is a novel HGF remains to be elucidated. With respect to the failure of hIL-3 to support the growth of IL-3 dependent murine cell lines, it has been demonstrated that human ^{125}I-IL-3 does not bind to factor dependent murine cell lines (FDC-P-2 and P8) known to express IL-3 receptors (Park et al., 1989a,b).

Production of hIL-3 by expression of the isolated hIL-3 cDNA has been successfully performed in prokaryotic as well as eukaryotic systems. Microorganisms used for the production of recombinant hIL-3 include yeast and bacteria such as *K. lactis*, *Bacillus* and *E. coli* species. The latter was used for the production of LacZ/hIL-3 fusion proteins which facilitated the production of antibodies directed against the hIL-3 polypeptide. With regard to these *E. coli* derived lacZ/hIL-3 fusion proteins, it is noteworthy that the fusion protein encoded by pUC/hMultiΔ2 (Fig. 3.12) expression vector lacks the first 14 N-terminal amino acids of the mature hIL-3 while remaining fully active. This hIL-3 mutant illustrated that the deletion at the N-terminus does not abolish its biological activity indicating that the deleted part is probably not involved in binding to the IL-3 receptor. However, it can not be excluded from these experiments that the heterologous lacZ part compensates for the function of the deleted 14 N-terminal residues of mature hIL-3.

These observations initiated studies on the structure-function relationship of hIL-3 by analyzing mutagenized proteins (Dorssers and Van Leen, 1991; Dorssers et al., 1991). The muteins with selected amino acid deletions as well as substitutions were engineered by site-directed mutagenesis. Generally, these studies will provide insight in important domains such as receptor binding sites, catalytic domain(s), presence of metal or cofactor binding sites and the antigenic determinants. From these studies it was concluded that indeed the first 14 N-terminal residues are not obligatory for biological activity. Another objective of these studies is the retrieval of agonists with improved therapeutical properties and specific antagonists of hIL-3 which are molecules that preclude signal transduction by binding to the IL-3 cell surface receptor, preferably with a high affinity. Obviously, such antagonists would have therapeutic potential,

especially, for the treatment of HGF-associated leukemias.

Large-scale production of hIL-3 accomplished by expression in e.g. B. *licheniformis* will facilitate a detailed analysis of its role in human hemopoiesis. The effects of recombinant human IL-3 on normal as well as malignant hemopoietic cells are presented in chapter 5. Furthermore, the availability of recombinant hIL-3 will enable *in vivo* studies in human and nonhuman primates to determine its therapeutic window and side-effects. The pleiotropic effects of murine IL-3 observed both *in vitro* and *in vivo* suggest a broad spectrum of therapeutic applications for hIL-3, which will be considered in chapter 7.

Yang et al. (1986) isolated a gibbon IL-3 cDNA by mammalian cell expression cloning. The gibbon IL-3 cDNA shares 91% homology with hIL-3 cDNA. It was shown that gibbon IL-3 supports the growth of human myeloid and erythroid progenitors *in vitro* (Leary et al., 1987).The gibbon IL-3 sequences enabled the isolation of the hIL-3 homologous genomic sequences by standard cross-hybridization methods (Yang et al., 1986; Yang and Clark, 1987). The sequence of human IL-3 mRNA and its encoded amino acid sequence were deduced from the genomic sequence. Comparison of this hIL-3 sequence as reported by Yang et al. (1986) with the sequence as presented in Fig. 3.8 revealed a T instead of the C at position 117 (see also Fig 3.5; pos 117). This nucleotide (nt) substitution results in an aa change (a proline to a serine) at position 8 of the mature protein (Fig. 3.10). The question as to whether this difference represents polymorphism of the hIL-3 gene has been investigated by several groups. A variety of cDNA clones encoding hIL-3 have been isolated from T cell libraries (Otsuka et al., 1988). These investigators reported a hIL-3 cDNA sequence identical to that shown in Fig. 3.8 . Furthermore, sequence analysis of four additional cDNAs, isolated independently from HG120 and 6D11 libraries (human Th cell clones), confirmed the C instead of T at the position in question. Thus, the sequence as presented in Fig. 3.8 as well as five independent hIL-3 sequences disclosed by Otsuka et al. (1988) showed at the vexed nucleotide substitution a C instead of T (proline instead of serine). Furthermore, Park et al. (1989a) reported the sequences of four hIL-3 cDNAs, isolated from a cDNA library constructed with mRNA extracted from peripheral blood T lymphocytes. All sequences encoded a proline instead of serine at position 8 of the mature hIL-3 protein, thereby confirming the aa sequence as presented in Fig. 3.10. Together, these investigators examined DNA from 13 different individuals for the presence of hIL-3 polymorphic DNA. Each of the 13 individuals contained DNA encoding proline at position 8 of the hIL-3 gene. However, three were also positive for serine at this position, suggesting polymorphism of the hIL-3 gene with the allele encoding proline being the most prevalent.

CHAPTER 4

MOLECULAR CLONING OF THE GENE ENCODING RHESUS MONKEY (*MACACA MULATTA*) INTERLEUKIN-3

Parts of this chapter have been published in:

Burger, H., R.W. van Leen, L.C.J. Dorssers, N.L.M. Persoon, P.J. Lemson and G. Wagemaker. Species specificity of human interleukin-3 demonstrated by cloning and expression of the homologous Rhesus monkey (Macaca Mulatta) gene. Blood. 76: 2229-2234, 1990.

Burger, H., L.C.J. Dorssers, R.W. van Leen and G. Wagemaker. Nucleotide sequence of the gene encoding rhesus monkey (Macaca mulatta) interleukin-3. Nucl. Acids Res. 18: 6718, 1990.

4.1 Introduction

The molecular cloning and expression of the gene encoding human IL-3 and the ensuing availability of large quantities of well-defined recombinant hIL-3, as described in the previous chapter or as reported by other investigators (Yang et al., 1986; Otsuka et al.,1988), facilitated extensive clinical study of this HGF. *In vitro* studies on normal human bone marrow cells revealed that hIL-3 functions as a multilineage hemopoietin (see also chapter 5) and exhibits the capacity to support the proliferation and development of multipotent cells (Barber et al., 1989) as well as the development of lineage restricted myeloid progenitor cells, including those committed to the erythroid, megakaryocytic, all granulocytic/monocytic and mast cell pathways (Dorssers et al., 1987; Bot et al., 1988; Lopez et al., 1988; Barber et al., 1989). Santoli et al. (1988) provided some evidence that the effects of hIL-3 are not entirely limited to the myeloid lineages since their data indicate that hIL-3 as well as GM-CSF supports the growth of cells within the lymphoid lineage and exerts potent amplifying effects on IL-2 induced T cell growth *in vitro*. Furthermore, recombinant hIL-3 has been shown to stimulate the proliferation of acute myelocytic leukemia clonogenic cells (Delwel et al., 1987/1988; Miyauchi et al., 1987; Vellenga et al., 1987b) as well as the proliferation of clonogenic progenitors derived from patients suffering from other bone marrow-associated disorders, including myelodysplastic syndromes, myeloproliferative disorders, or the B cell-associated multiple myeloma (Barber et al., 1989; Bergui et al., 1989). With regard to IL-3 responsiveness of malignant progenitors, marked variations between individual patients were observed, which may reflect growth factor requirements of different differentiation stages of normal bone marrow cells. The pattern of the growth factor requirement of a neoplastic clone may be additive to the morphological classification (FAB) of leukemic cells. In summary, the broad spectrum of biological activities of hIL-3 observed *in vitro* is similar to that observed when mouse bone marrow was stimulated by murine IL-3 (reviewed by Ihle et al., 1989). In addition, effects observed in *vitro* with recombinant gibbon IL-3 on human bone marrow (Yang et al., 1986; Leary et al., 1987; Lopez et al., 1987; Sieff et al., 1987b) further indicate that primate IL-3 is functionally similar to murine IL-3.

The large-scale production of recombinant hIL-3 has also prompted preclinical *in vivo* studies in monkey species to identify its therapeutic window and side-effects (Donahue et al., 1988; Krumwieh and Seiler, 1989; Mayer et al., 1989; Umemura et al., 1989). Contrasting its broad range of action *in vitro*, recombinant human IL-3 administered to rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca facsicularis*) exerted only limited and in part inconsistent effects on the production of peripheral blood cells. In fact, the effects of hIL-3 on peripheral blood cell numbers in these *Macaca* species were restricted to a small rise in white blood cell numbers, predominantly caused by

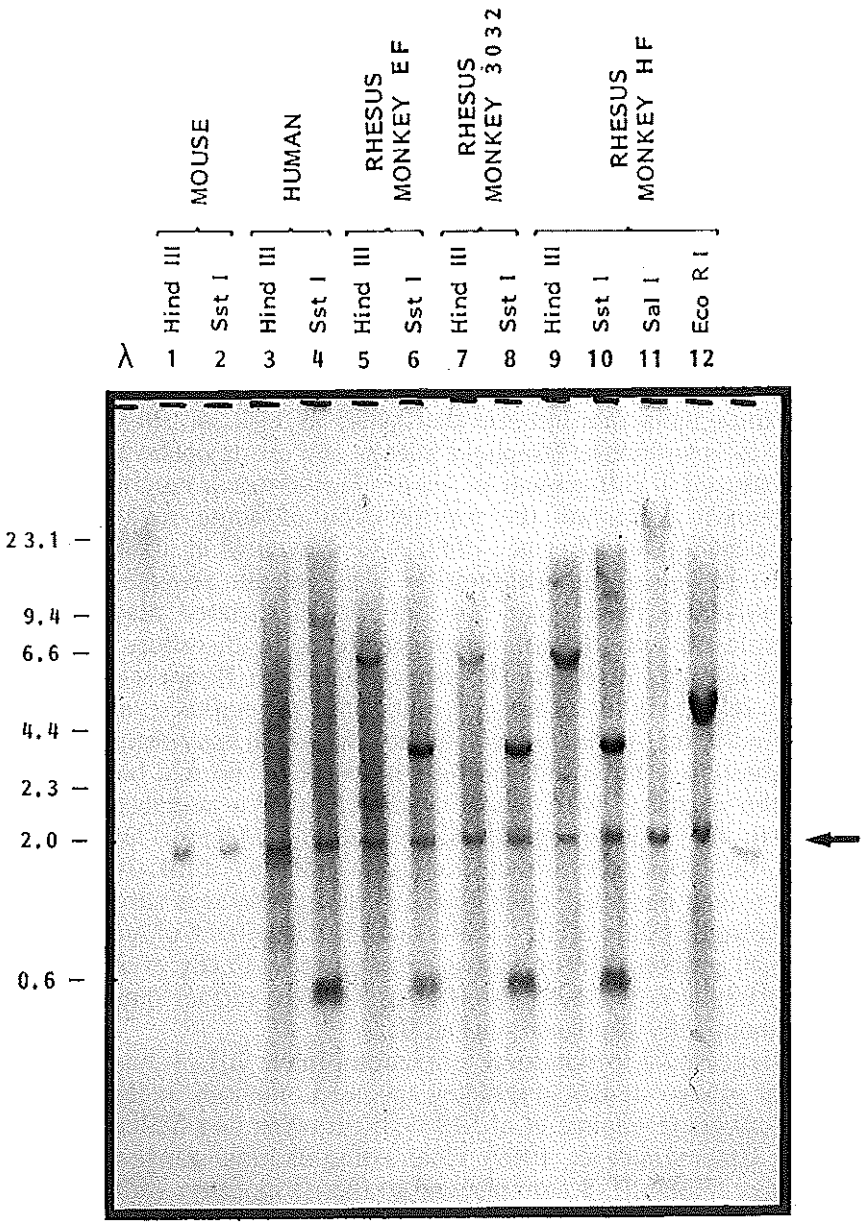
λ
1 Hind III
2 Sst I
MOUSE
3 Hind III
4 Sst I
HUMAN
5 Hind III
6 Sst I
RHESUS MONKEY EF
7 Hind III
8 Sst I
RHESUS MONKEY 3032
9 Hind III
10 Sst I
11 Sal I
12 Eco R I
RHESUS MONKEY HF
23.1
9.4
6.6
4.4
2.3
2.0
0.6

Fig. 4.1 Southern blot analysis of mouse, human and rhesus monkey chromosomal DNA. Genomic DNA (8 µg) from mouse (lanes 1 and 2), human (lanes 3 and 4), and three individual rhesus monkeys (lanes 5-12) were digested with the indicated restriction enzymes and separated on a 0.6% agarose gel. DNA fragments were transferred onto a nylon membrane (GeneScreen Plus, NEN Research Products, Boston) and hybridized to random-primer labeled hIL-3 cDNA lacking 3' the noncoding region (nt 1-541; see Fig. 3.5). Molecular weight markers (Lambda DNA digested with *Hind*III) are shown at the left. The arrow indicates a nonspecific fragment (present in all samples) hybridizing with the hIL-3 probe and presumably represents vector DNA sequences.

basophilia. Somewhat larger effects of hIL-3 were noted by sequential administration of other, more pathway-restricted hemopoietic growth factors, such as granulocyte/macrophage colony-stimulating factor (GM-CSF) or erythropoietin (Donahue et al., 1987; Umemura et al., 1989). Therefore, it was generally held, that IL-3 expands an early cell population that subsequently requires the action of later acting factors such as GM-CSF to complete its development (Donahue et al., 1987). In contrast to this hypothesis, the first clinical trial of recombinant hIL-3 in human patients showed elevated numbers of neutrophilic and eosinophilic granulocytes as well as substantial rises in thrombocytes.

Based on such evidence, we proposed an alternative hypothesis that the limited effects of human IL-3 in *Macaca* species should at least partly be attributed to its species specificity. To test this hypothesis, we decided to clone and express the gene encoding rhesus monkey IL-3 (RhIL-3) and to study the stimulatory activity of the encoded RhIL-3 protein as well as the homologous human protein on human and rhesus monkey cells.

Southern blot analysis revealed that hIL-3 cDNA hybridizes with genomic rhesus monkey DNA (Fig 4.1), which suggests that the RhIL-3 gene can be readily isolated by screening a genomic rhesus monkey library with the hIL-3 cDNA. A genomic DNA library should contain stable DNA sequences representative of the entire rhesus monkey genome and inserts should be large enough to contain the whole RhIL-3 gene with its flanking regions. Furthermore, it should be possible and simple to retrieve the inserted DNA from the vector sequences in order to introduce it into sequence and expression vectors. Amplification of the library without loss or misrepresentation requires the generation of sufficient individual recombinants for a "complete" library. Genomic libraries that fulfill these requirements appeared to be available. A lambda (λ) Charon 32 as well as a λ Charon 40 library, containing partially digested *Eco*RI genomic rhesus monkey DNA were provided by Dr. Jerry L. Slightom (Kalamazoo, Michigan, USA). These lambda Charon phages permit restricted size inserts of approximately 20 kb due to packaging limitations. The genes encoding mouse (Miyatake et al., 1985), rat (Cohen et al., 1986) and human IL-3 (Yang and Clark, 1987) are relatively small genes approximately 3

kb in length. The λ Charon libraries mentioned above, which represent approximately 90% of the entire rhesus monkey genome, were thought to be appropriate for successful isolation of the gene encoding RhIL-3.

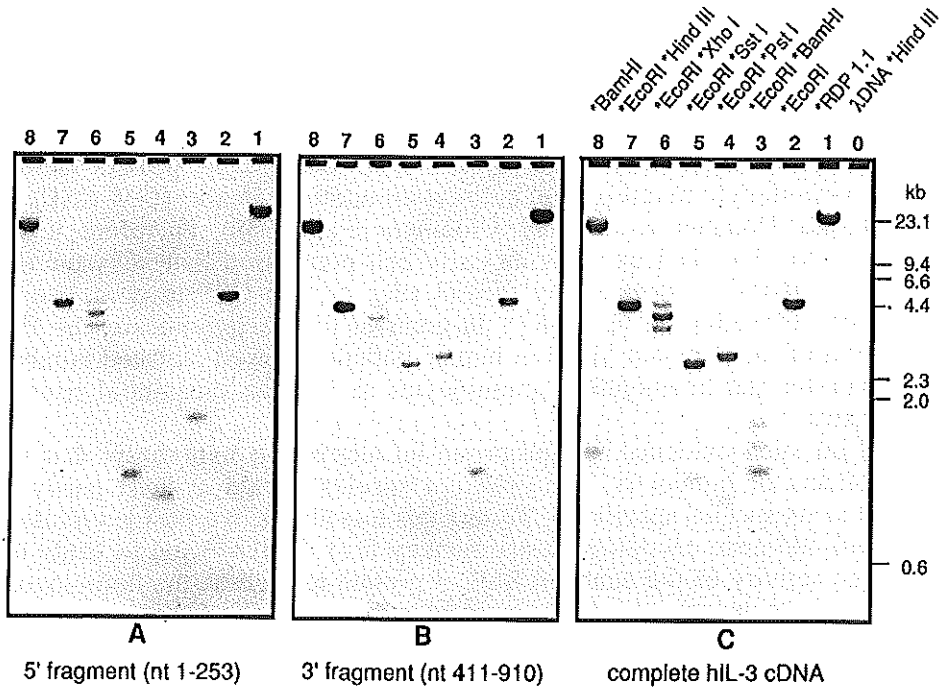

Fig. 4.2 Southern blot analysis of phage DNA isolated from clone RDP1.1. Phage DNA from clone RDP1.1 (lane 1: not digested) was digested with the indicated restriction enzymes and separated on a 0.8% agarose gel. DNA fragments were transferred onto nylon membranes (GeneScreen Plus, NEN Research Products, Boston) and hybridized to different hIL-3 cDNAs (see Fig. 3.5 for nt positions) as indicated (Panel A-C). Molecular weight markers (Lambda DNA digested with *Hind*III) are shown at the right.

4.2 Isolation and characterization of the rhesus monkey IL-3 gene

About 10^6 plaques of the lambda Charon 40 library were screened with

radiolabeled hIL-3 cDNA as described in chapter 2 and a single positive clone (RDP1.1) was isolated. Analysis of phage DNA of clone RDP1.1 by restriction enzyme cleavage mapping showed that this clone contains an insert of approximately 13 kb with one internal *Eco*RI site, dividing the insert in 4.1 kb and 9 kb *Eco*RI fragments. Southern blot analysis revealed that the hIL-3 homologous sequences were located on the 4.1 kb fragment (Fig. 4.2C). The *Eco*RI site present in exon V of the hIL-3 gene was apparently not conserved in rhesus monkey, since both the 5'- and 3' fragment of the hIL-3 cDNA hybridized to this 4.1 kb fragment (Fig. 4.2A and 4.2B). The hybridization patterns of the 4.1 kb fragment, digested with different restriction enzymes (Fig. 4.2), indicated that in comparison to the human gene various restriction sites have been conserved and that the overall gene structures of RhIL-3 and hIL-3 are virtually identical, as shown in Fig. 4.3. Furthermore, comparison of the generated restriction endonuclease map of the 4.1 kb fragment (Fig. 4.3) with the hIL-3 genomic sequence (Yang and Clark, 1987) suggests that the 4.1 kb *Eco*RI fragment comprises all RhIL-3 coding sequences.

The 4.1 kb *Eco*RI fragment was subjected to restriction endonuclease digestion with *Ava*I and *Bam*HI. The generated fragments were subcloned into pTZ18R and subsequently sequenced in both directions as described in chapter 2. Oligonucleotides, complementary to the established sequences, were synthesized to serve as internal primers for sequencing over the restriction sites used for subcloning. Computer-assisted analysis of the sequence data resulted in the complete nucleotide sequence of the 4.1 kb fragment as shown in Fig. 4.4. The sequence has been deposited at the EMBL Data Library with assignment of the following accession number: X51890 [*M. mulatta* interleukin-3].

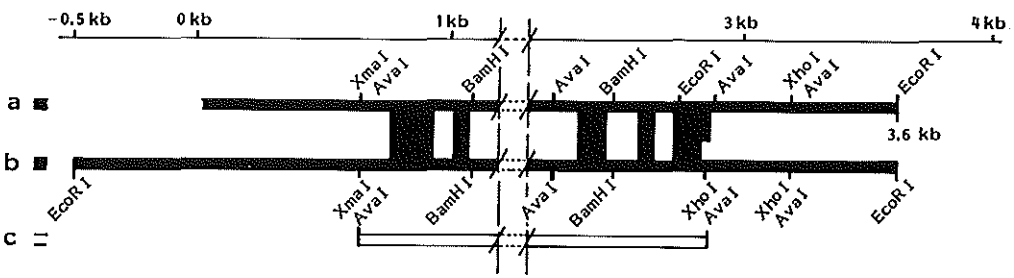

Fig. 4.3 Schematic representation of the genomic organization of the human and rhesus monkey IL-3 genes. Human IL-3 sequences (a; Yang et al., 1987) were aligned to the corresponding rhesus monkey IL-3 sequences (b). Black boxes indicate the protein coding sequences. Restriction sites used for sequence analysis are indicated. The 2.2 kb *Xma*I-*Xho*I RhIL-3 fragment (c) was used for transient expression in COS cells.

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GAATTCTTCCACATGCCCCCATCACACTCATCCCTTTACCAAAAGTCCCT      50
ACCCCATGGGGTGGGTCAGGCAGGCCTCAACACAGGCCCGTATCAGGGGG     100
ACGCCTCTTCCCTCAGGGGCTGCCCTCTGGGATCACCACCCCCGCCCTCT     150
TTGGTTTCCTGCTTCCTATCTGGCTGCAGCTTTTCAGGCCCATTGTGTAT     200
TTCCCCATGGTCTGTGCCCACTCGCATCCCCTCTCTGCAAACCTTGCCTA     250
CTGGGCCTGTACCTGGCAAATCCATGCTCAGCACAGACAGGGATGGAGAC     300
CTCTCAATACAACTGTCTCCTGCCAATCCCTGCCCCAGCAGCCTGAGGCC     350
CAGCCTGCAACCAGGGAGCTGCTCTCCTTTCTCCTCTCCTCCCTTGACCT     400
CGCCCCTCAGACCATGCCAATTCTACCTCCTAAACCTCCCAGGCCAGCCC     450
CTCCCCCAGCTCCCAGTGACAGTGTCCTCAAGAGCTCCTGTCCTTAGGTA     500
CCTGAGCTCAGCTCTTGGTGATACCAGAAGTACTGCCAGGGGCTGCAGCA     550
GGCCTCCTGCAGAGGCTGAGTCCCACATCGCAGGGAACAGCCATGCCACT     600
GCTAGCAGACAAGTAAGAGAATGGCCACCTGGGGCTGAGTGCTCTTGGTC     650
ACCCACCAGAAACAAAGTGTCAAGGAGAAGCTGCCCAAAGCCCATGGGAC     700
AAACCACTGGGGATTGGAACACCAGTAATTCCGCATTGGGAGGGGGCACC     750
AAGAGATGTGCTTCTCAGAGCCTGAGGCTGCACATGGATGTTGAGCAGTG     800
CGACCGGCTACCAGACAAACTCATCAGTTCCAGTGGCCTCCTGGCCACCA     850
CCAGGACCAAGCAGGGTGGGTAGCAGAGGGCCACCTGGAGGTGATGACAG     900
ATGAGATCCCGCTGGGCAGGAGGCCTGAGTGAGCTGAGTCAGGCTTCCCC     950
TTCCTGCCACAGGGGTCCTCTCACCTGCTGCCATGCTTCCCATCTCTCAT    1000
CCTCCTTGACAAGATGAGGGGATACCCTTTAAGTAATCTTTTTTCTTGTT    1050
TCACTGATCTTGAGTACTAGAAAGTCATGGATGAATAATTACGTCTGTGG    1100
TTTTCTATGGAGGTTCCATGTCAGATAAAGATCCTTCCGACGCCTGCACC    1150
ACACCACCCGCCCCCCGCCTTGCCCGGGGTTGTGGGCACCGTGCTGCTGC    1200
ACATATAAGGCGGGAGGCTGTTGCCGACTCTTCAGAGCCCCACGAAGGAC    1250
CAGAACAAGACAGAGTGCCTCCCACCAATCCAAACATGAGCCGCCTGCCC    1300
                                    M  S  R  L  P
GTCCTGCTCCTGCTCCATCTCCTGGTCAGCCCCGGACTCCAAGCTCCCAT    1350
V  L  L  L  L  H  L  L  V  S  P  G  L  Q  A  P  M
GACCCAGACAACGTCCTTGAAGACAAGCTGGGCTAAGTGCTCTAACATGA    1400
 T  Q  T  T  S  L  K  T  S  W  A  K  C  S  N  M  I
TCGATGAAATTATAACACACTTAAACCAGCCACCTTTGCCTTCACCGGTG    1450
   D  E  I  I  T  H  L  N  Q  P  P  L  P  S  P
AGTAGCTGGGATAAGACTGGCCTGCAGCAGGGAGGGGTGGTGGCTGCCTC    1500
AGCCAACAGCCTCATGGGCCTCTCTCTCCCTTCACCCCATAGGACTTCAA    1550
                                          D  F  N
CAACCTCAATGAGGAAGACCAAACCATTCTGGTGGTAAGAGCTCAGCCCC    1600
 N  L  N  E  E  D  Q  T  I  L  V
TGGATCCCAATCCACTTCCTGCCTGGGTGACTTCAGCCATGTCATTCCAT    1650
CTTGCCTAGCCTTGCTTTCTTCATCTGTAAAATAGGGTTAATAGCGCCTG    1700
TCTCAGTGGGACTGTTATGACAGTCAAATGACACAATGTGCATAACGTTC    1750
TGGCCCAGCATCTGGCACTTAAGGGTTCAATACGTGGCCACAGCCATGGC    1800
TATAAGAATGAAAATGACTTTTAAATTAGAAAATGAAAAGCCCCACAGAT    1850
GTGCCAGCTTCTTTTTCCCTTTAAAAAGTGAGTTTGTGTTTTCCACCCTC    1900
ATTGGTTGTGGCATTATCATTTACAGTTATTTTTCATGGTTCCAGTTGGT    1950
TGCAATGGTTCTCTCTCACGTTTGCATGACATTTCTTGGGTCAAACAAGA    2000
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TCCTGGGCCATCTGTATTTGGGGCACTCACTGCTCACATGCTGCACCCAC    2150
TTCCATGTTGCGGTCTCTGTGATTTTCTTCTTGATTTCTAGGAGACTTTA    2200
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CCCATGGAAGACCCCCTGCCTTTTGTAGAGTTTCTTTTTGGAGATATGTT    2300
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TTTTTTTTTTGAGACAGAGTCTCACTCTGTTGCCCAGGCTGGAGTGCAGT    2400
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GGCACGATCTTGGCTCACTGCTACCTCTGCCTCCCAAGTTCAAGCCATTC  2450
TCCTGCCTCAGCCTCCCAAGTAGCTGGGACTACAGGCATGCACCACCACT  2500
CCCATTTTTGTATTTTTAGTAGAGTCGGGCTTTCACCATATTGGTCAGGC  2550
TGGTCTTGAACTCGACCTCAGGTTATCTGCCTGCCTCGGCTTCCCAAAAT  2600
GCTAGGATTACAGGCTTGAGTCACCGCACCCGGCCCACCTAGGCCCTTCA  2650
GGCAGCTCACATGGAGCCAGAGACTGGGGGCTTGAGGAAACCAGGTCCTG  2700
CCTGCCACTCACTTCTAGGCCTGTGCTCTTGGGCAGGGACCCACCTGAGG  2750
CAAGAATGGGACTAGGAGGGAACCCGAGAGTGGGCTTGGGAAACCCTGGG  2800
GGTGACTTCCAGCTGCTTGTGGGAGGGATACTCCGTAACCTTTCCCCCGT  2850
AAGTGTATTCTCTGCCCTGTTAGGAAAAAAACCTTCGAAGGTCAAACCTG  2900
                          E  K  N  L  R  R  S  N  L
GAGGCATTCAGTAAGGCTGTCAAGAGTTTACAGAACGCATCAGCAATCGA  2950
E  A  F  S  K  A  V  K  S  L  Q  N  A  S  A  I  E
GAGCATTCTTAAGGTATGTGAAGCTGTTGAGGGTTTGGGATCCCTGTGTT  3000
 S  I  L  K
GGCCCTGCCCTGCCTCTGGGGAGGATGACAGCAGGGCCCACTCCCTTTCC  3050
AAGGGAATCTCTGACCATCTGCTTTGATCTCTTTCCACAGAATCTCCCAC  3100
                                                 N  L  P  P
CATGCCTGCCCATGGCCACGGCCGCACCCACGGTAAGCTGTCCCCCAAGA  3150
  C  L  P  M  A  T  A  A  P  T
TGCCCGTGTCGTGGCTTGCTCCTCAGTTGGTCATCACCATTATAGCCTGG  3200
ACTCACCTAATCCCACCTTCTTGGTTTCTTTATAGCGACCTCCAATCCGT  3250
                                          R  P  P  I  R
ATCACGAACGGTGACCGGAATGACTTCCGGAGGAAACTGAAGTTCTATCT  3300
I  T  N  G  D  R  N  D  F  R  R  K  L  K  F  Y  L
GAAAACCCTTGAGAATGAGCAGGCTCAATAGACGACTCTGAGCCTCGAGA  3350
 K  T  L  E  N  E  Q  A  Q  *
TCTCTTGAGTCCAACGTCCAGCTCGTTCTCTGGGTCTTCTCACCACAGAG  3400
CTTCAGGACATTGAAAACTTCTGAAACCTCTGGCTCATCTCTCACACAGT  3450
CCAGGACCAGAAGCATTTCACCTTTTCCTGCGGCATCAGACGAATTGTCA  3500
ATTATCTAATTTCTGAAATGTGCAGCTCCCATTTGGCCTTGTGCAGTTGT  3550
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GACTTAGTGACCATTTTAGAGGAGACTGGTGTGCCACGAATCCTGGGTGG  3850
CTTGATCCTGCCACGTGGATGTTGTCTGTGTGAGCTTGTTCTCACACTGC  3900
CCTCCTGCCACCCCCATTTCCAGAAAGGTGATGATAACCCTAGCAATCTT  3950
GAATATCCACAGAACTGCTACCAGGTACCAGGAGCCATTCTCAGCACTTT  4000
ACCTGTGCTACCTAACTTACTCCTCACACCAACCAAGAGTATGTTTTCTC  4050
CGTTTCATGGGAAACTGAAGTTCAGCGTAGCTGAGCAACTGCCTAAGGTC  4100
ACACAGCTGATAATGGCCCAGCTGTGGCTTGAATTC                4136
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Fig. 4.4 Rhesus monkey genomic DNA sequence encompassing the IL-3 coding regions. The nucleotide sequence of the 4.1 kb EcoRI fragment of RDP1.1 is shown and the deduced RhIL-3 protein sequence is denoted in the single letter code indicated under the thirst nt of the corresponding codon. The numbers at the right refer to the last nt of the corresponding line.

The nucleotide sequence of the entire 4.1 kb *Eco*RI fragment (Fig. 4.4) compared with the complete sequence (3600 bp) of the hIL-3 gene (Yang and Clark, 1987) displayed a homology of 92.9%. An identical percentage of homology was found for the coding regions. The intron/exon boundaries, specified by a consensus splice donor and acceptor sequence, were found to be highly conserved. The 5'-flanking region upstream the putative transcription initiation site (Fig. 4.5A pos. 1234) presumably spans the putative promoter and contains structural elements common to many eukaryotic promoters (Benoist and Chambon, 1981; Fromm and Berg, 1982; Treisman et al., 1983) including a "TATAA"-related sequence (Fig. 4.5A pos. 1204), a "CAT"-related sequence (Fig. 4.5A pos. 1115) and a G+C rich sequence separating these elements. Furthermore, one copy of the conserved "decanucleotide" with the consensus "GPuGPuTTPyCAPy" (Fig. 4.5A pos. 1110, denoted as CK-1 and overlapping the CAT box) present in the 5'-untranslated regions of many HGFs (Stanley et al., 1985; Kaushansky, 1987; see also chapter 1) and a sequence located at the 3' end of the decamer (Fig. 4.5A pos. 1121, denoted as CK-2) with the consensus "TCAGPuTA" (Shannon et al., 1988) were identified in the promoter region of the RhIL-3 gene. All closely linked "upstream promoter elements" and their relative position, as identified in the RhIL-3 gene, are conserved in all analyzed IL-3 species. Furthermore, sequence comparisons were conducted in order to identify other closely or more distantly located sequences required for efficient regulation of IL-3 expression by binding transcription factors. Potential binding sequences of the transcription factors (reviewed by Wasylyk, 1988, Mitchell and Tjian, 1989, Yoza and Roeder, 1990) or enhancers such as Sp1, AP-2, SRF, OTF, OCT-1, N element, heptamer, CR† and CREB were not found. However, some distinct binding sites for sequence-specific transcription factors, including AP-1 (Mathey-Prevot et al., 1990), CTF/NF-1 (Mitchell and Tjian, 1989) and NF-κB (Lenardo and Baltimore, 1989) were identified upstream of the putative transcription initiation site (Fig. 4.5A pos. 1235) of the RhIL-3 gene. Two adjacent AP-1 sites (Fig. 4.4 pos. 926 and 935) and a NF-κB site (Fig. 4.4 pos 1018) were present in the vicinity of the transcription start site (-300 to -200 bp relative to the transcription initiation site), whereas two copies of the CTF/NF-1 site (Fig. 4.4 pos. 322 [-900 bp] and 416 [-800 bp]) were located more distant relative to the position of RNA chain initiation.

The 3' noncoding region upstream the polyadenylation site (Fig 4.5B pos. 3746) contained 8 "ATTTA" repeat units. With respect to their position, five of these repeats are conserved in the hIL-3 gene (Yang and Clark, 1987; Dorssers et al., 1987). The significance of the presence of these highly conserved AU-rich sequences in genes encoding various cytokine and their postulated role in the post-transcriptional regulation by the modulation of translational efficiency have been discussed in the previous chapter.

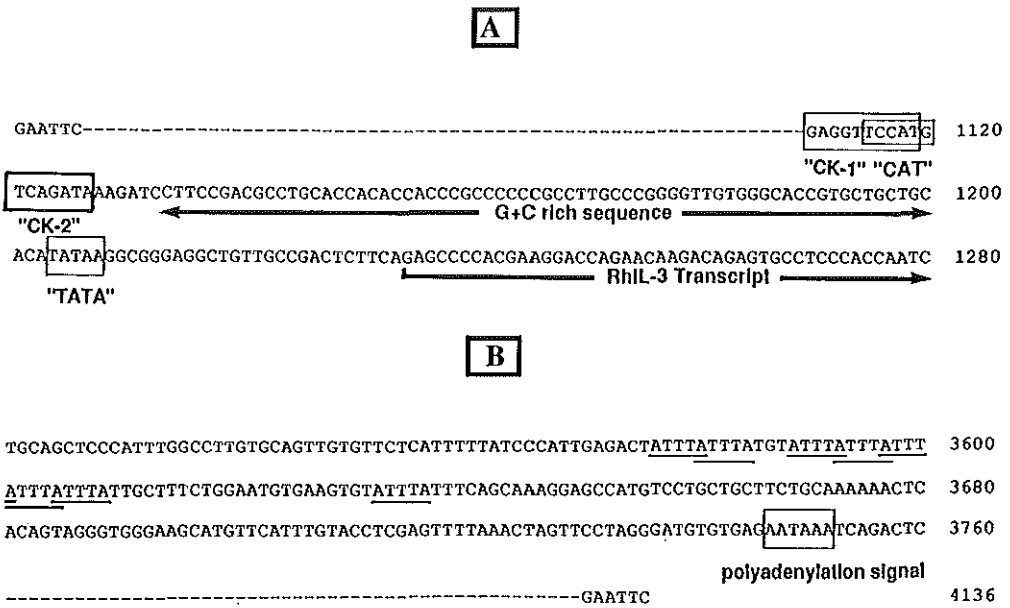


Fig. 4.5 Structural elements in the 5'- and 3'-flanking regions of the RhIL-3 gene. The 5'-flanking sequence upstream of the transcription initiation site (panel A) and the 3' noncoding region upstream of the polyadenylation site (panel B) are shown. The sequence shown in panel A spans the putative promoter region and contains several regulatory elements including a TATA box, a CAT box, a G+C rich sequence and the CK-1 (Stanley et al., 1985) and CK-2 (Shannon et al., 1988; Mathey-Prevot et al., 1990) motifs. The polyadenylation signal and eight "ATTTA" motifs present in the 3' noncoding region of the RhIL-3 gene are indicated in panel B.

The intron/exon structure of the RhIL-3 gene, deduced from the nucleotide sequence of the hIL-3 cDNA and confirmed by sequence analysis of the isolated RhIL-3 cDNA construct, shows that the RhIL-3 gene, like the murine, rat and human counterparts (Miyatake et al., 1985; Cohen et al., 1986; Yang and Clark, 1987) is divided into five small exons, encoding in this case a 143 amino acid polypeptide. This RhIL-3 polypeptide is most likely processed at the amino terminus by the removal of 19 residues to yield a mature protein comprising 124 amino acids (M_r 13,974) , which is 9 residues shorter than the human protein (M_r 15,074). The hIL-3 and RhIL-3 protein homology was found to be 89.5% for the signal peptide and 80.5% for the mature protein as shown in Fig. 4.6. The two cysteine residues at position 16 and 84 present in hIL-3 are conserved in the rhesus monkey (Fig. 4.6) and may play an essential role in

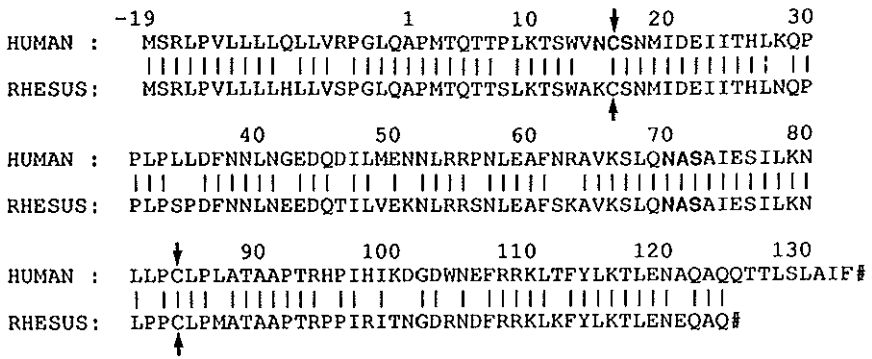


Fig. 4.6 Alignment of the aa sequences of rhesus monkey and human IL-3. The protein sequences are shown in the single letter code and identities are indicated by vertical lines. The two conserved cysteine residues are indicated by arrows and the potential N-linked glycosylation sites are shown in bold.

protein folding. The potential N-linked glycosylation site present at position 70 of hIL-3 (Fig. 4.6) is conserved in RhIL-3 but the potential glycosylation site at position 15 is deleted due to substitution of the asparagine residue in hIL-3 by a lysine in RhIL-3 (Fig. 4.6). Comparison of the IL-3 coding sequences of man and rhesus monkey, as shown in Fig. 4.7, revealed that the majority of substitutions, approximately 80%, had occurred at replacement or nonsynonymous sites of codons resulting in a change of the amino acid sequence (Li et al., 1985). This aspect has been further investigated and the results are presented and discussed in chapter 6.

4.3 Expression of RhIL-3 coding sequences in COS cells

The 2.2 kb *Xma*I-*Xho*I (filled in) blunt fragment of RDP1.1 (Fig. 4.3) was used for expression in COS cells. This fragment, comprising all hIL-3 homologous coding sequences, was inserted in the *Sma*I site of the polylinker downstream the heterologous SV40 late promoter in the pSVL (Pharmacia) eukaryotic expression vector, resulting in the pSVL/RhIL-3 plasmid (Fig 4.7). Plasmid DNA was purified and transfected, as described in chapter 2, to COS cells, which permit efficient replication of plasmids carrying a SV40 replication origin (Gluzman, 1981). Supernatant was harvested 48-72 hours after transfection and tested for the presence of functional RhIL-3 by the capacity of the supernatant to stimulate hemopoietic cells. Western blot analysis using polyclonal as well as monoclonal antibodies directed against the hIL-3

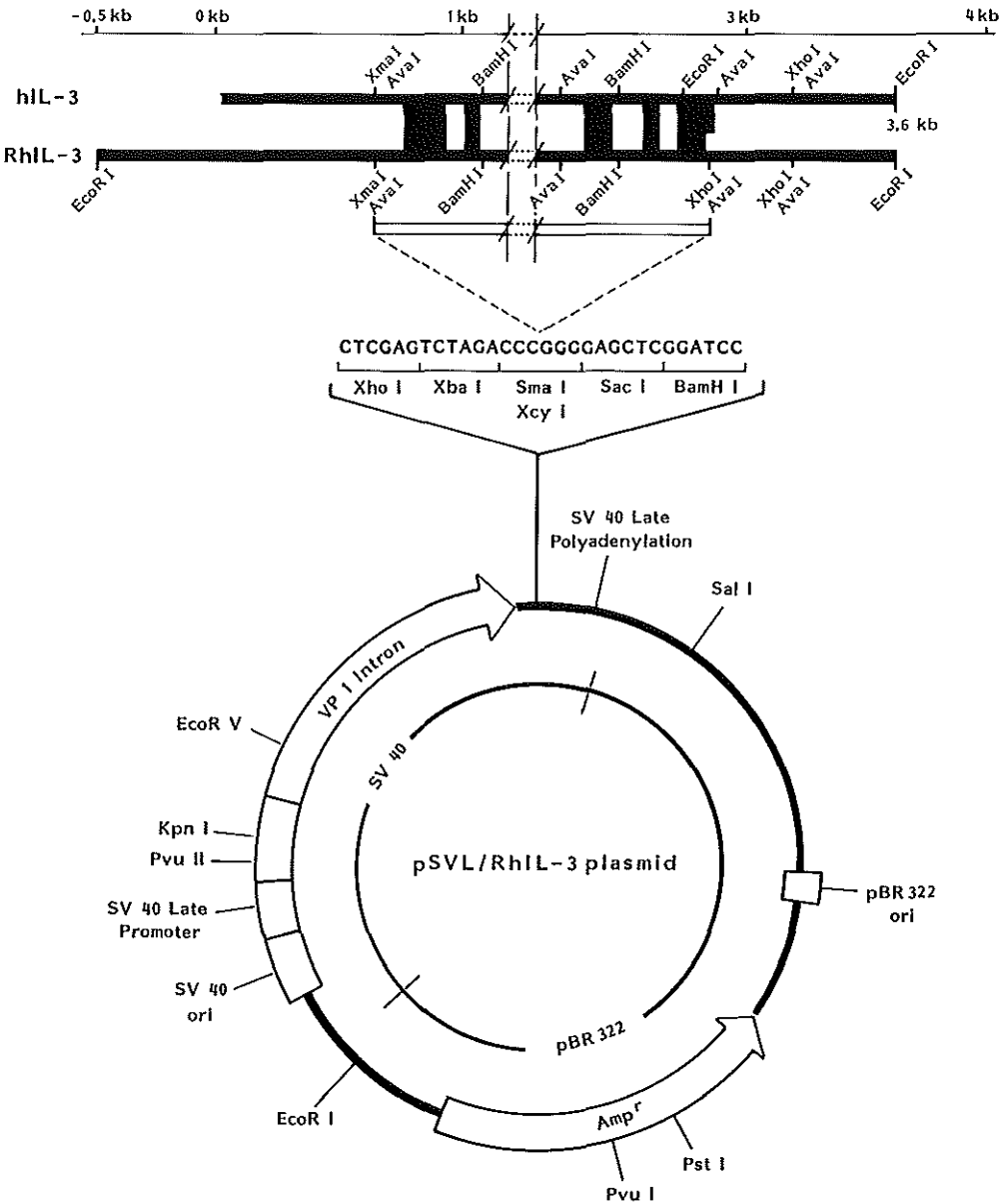


Fig. 4.7 Structure of the pSV/RhIL-3 expression vector. The 2.2 kb *Xma*I-*Xho*I (filled in) RhIL-3 fragment of RDP1.1 (see Fig. 4.3) was inserted into the *Sma*I site of the

polylinker of the pSVL (Pharmacia) eukaryotic expression vector resulting in the pSV/RhIL-3 vector (see chapter 2). The SV40 late promoter drives the expression of the inserted RhIL-3 gene.

polypeptide demonstrated that these antisera immunoreacted with the RhIL-3 protein. The number of IL-3 units present in the supernatant was assessed on IL-3 dependent human AML-193 cells as well as on rhesus monkey bone marrow progenitor cells and appeared to be in both assays approximately 100 units per ml. The biological data of the COS cell-derived RhIL-3 will be presented and discussed in the next chapter. Purification and assessment of the specific activity of RhIL-3 expressed in COS cells were not attempted because of the relatively low expression level. As shown in the previous chapter, large-scale production of hIL-3 was accomplished by prokaryotic expression. Therefore, the construction of a RhIL-3 cDNA, required for the synthesis of RhIL-3 protein in bacteria, was initiated. Such a production system will facilitate *in vivo* studies on homologous IL-3 in rhesus monkeys.

4.4 Construction and isolation of a rhesus monkey IL-3 cDNA

Poly A+ mRNA was isolated from COS cells 48 hours after transfection with pSVL/RhIL-3 and 2 µg was converted to single stranded cDNA using oligo (dT), as described in chapter 2, for the first strand reaction. The generated single stranded cDNA was selectively amplified with the help of the polymerase chain reaction (PCR) technique using oligonucleotides designed for RhIL-3 cDNA cloning into a *Bacillus* expression vector. The first oligonucleotide has the sequence 5'-TGC GAC GTG GAT CCT GCA GCA GCG GCG GCT CCC ATG ACC CAG ACA ACG-3' and contains *Bam*HI and *Pst*I restriction sites and the information for the 3' part of the *B. licheniformis* alpha-amylase signal peptide precisely fused to the mature RhIL-3 sequence. The second oligonucleotide has the sequence 5'-GGA TCC GAG CTC CCC-3' and is complementary to the *Sma*I-*Bam*HI fragment of the multiple-cloning site present in pSVL/RhIL-3 (Fig. 4.7) and concomitant complementary to the 3' end of mRNAs synthesized from pSVL constructs.

The reaction products of the PCR, carried out as described in chapter 2, were digested with *Bam*HI and *Xho*I and the resulting fragments were inserted into pGEM-7Zf(+) (Promega). The resulting plasmids were propagated in *E. coli* and individual transformants were, without prior hybridization selection, directly subjected to DNA sequence analysis. Since Taq-polymerase induces mutations due to incorrect nucleotide incorporation, the nucleotide sequence of cDNA inserts of several individual transformants were established as described in chapter 2. Comparison with the genomic sequence enabled the selection of a clone without induced mutations. In addition, these analyses revealed that the frequency of mutations induced by the PCR amplification technique, using Taq-

polymerase, was approximately 1 per 400.

4.5 Expression of RhIL-3 cDNA and large-scale production of the encoded RhIL-3 polypeptide

The selected insert of a pGEM-7Zf(+) clone, carrying the correct RhIL-3 cDNA as determined by sequence analysis, was cloned into an appropriate *B. licheniformis* expression vector similar to hIL-3 cDNA as described in chapters 2 and 3. *E. coli* as well as *B. licheniformis* vectors, which were used to acquire the definitive RhIL-3 expression vector, are described elsewhere (Persoon and Van Leen, 1990). In the resulting RhIL-3 *B. licheniformis* vector pGB/RhIL-326, the expression of the α-amylase/mature RhIL-3 open reading frame is driven by the strong *Hpa*II promoter. *Bacillus* strain T9 was transformed with pGB/RhIL-326 as described in chapter 2 and transformants were shown to produce high amounts of RhIL-3 (>5 mg/l) of which more than 95% was secreted into the culture medium. Purification of the secreted RhIL-3 polypeptide was performed analogous to hIL-3 (see chapter 2 and 3; and Persoon and Van Leen, 1990) with the exception that for the ion-exchange chromatography step, DEAE Sepharose Fast Flow (Pharmacia) was used instead of Fractogel TSK DEAE-650.

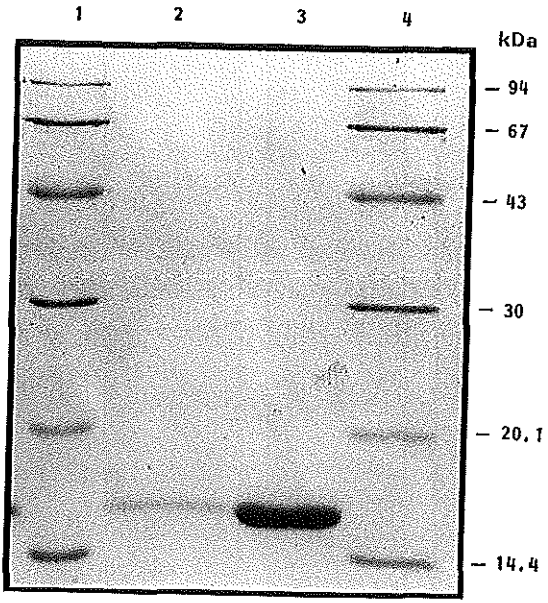


Fig. 4.8 Analysis of purified *B. licheniformis*-derived RhIL-3 by SDS-PAGE. Lanes 2 and 3 were loaded respectively with 0.2 µg and 2 µg protein of a purified RhIL-3 preparation (RhIL-3 IIIB). The single Coomassie brilliant blue stained band demonstrated that the RhIL-3 preparation has a purity of ≥99%. Lanes 1 and 4 were loaded with protein markers (LMW, Pharmacia) and their corresponding molecular weights are indicated in kDa at the right.

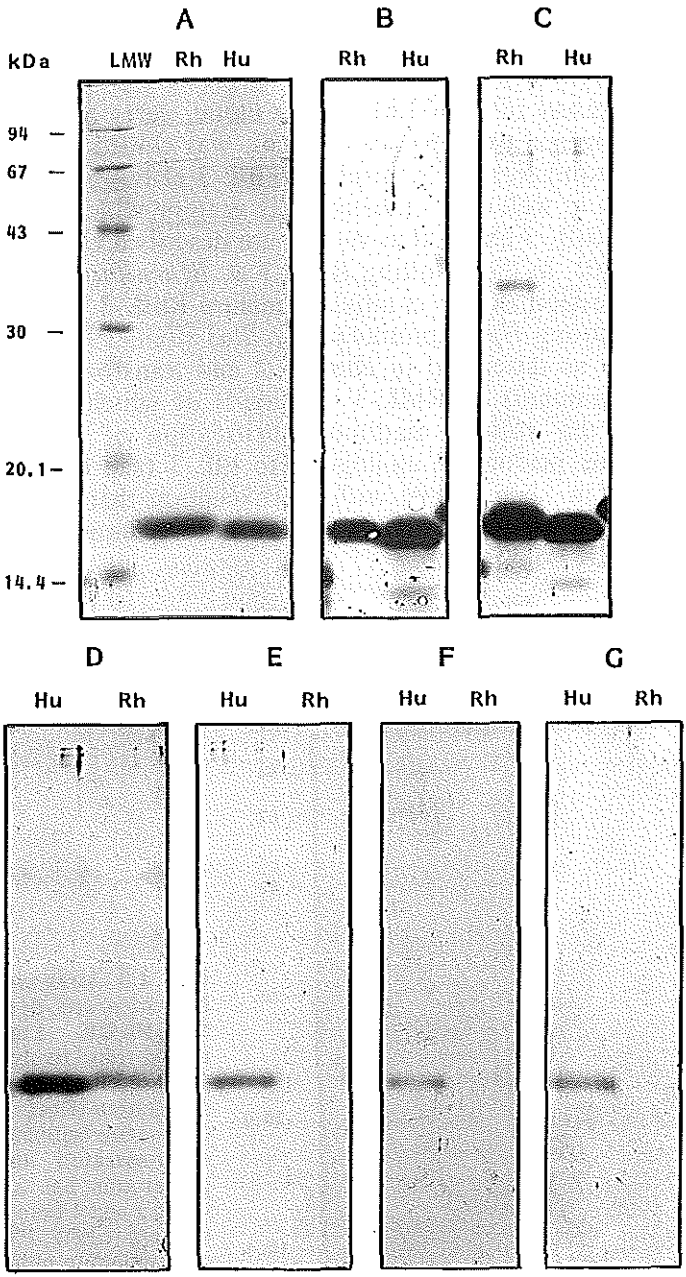

A
B
C
kDa
LMW
Rh
Hu
Rh
Hu
Rh
Hu
94
67
43
30
20.1
14.4
D
E
F
G
Hu
Rh
Hu
Rh
Hu
Rh
Hu
Rh

Fig. 4.9 Western blot analysis of human and rhesus monkey IL-3. Homogeneous *B. licheniformis* derived human and rhesus monkey IL-3 were used in these studies. Panel A shows coomassie brilliant blue stained SDS-PAGE of human IL-3 (Hu) and Rhesus monkey IL-3 proteins. Equal amounts of protein were loaded as demonstrated by comparable staining. Following SDS-PAGE (13.5%) IL-3 proteins (equal amounts of Hu and Rh) were transferred onto nitrocellulose membranes using the semi-dry transfer technique (see chapter 2). The filters were incubated with polyclonal rabbit anti-human IL-3 serum (panel B) and various MAbs directed against human IL-3 (see chapter 3; Dorssers et al., 1991): MCA/A4 (panel C), MCA/A8 (panel D), MCA/A 1 (panel E), MCA/A5 (panel F), and MCA/A18 (panel G).

The two step purification protocol, as described in chapter 2, resulted in a homogenous RhIL-3 preparation, as demonstrated by Western blot analysis using polyclonal and monoclonal antibodies (MCAs) directed against hIL-3 (see chapter 3) and by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which showed the presence of a single Coomassie brilliant blue stained band with a purity of $\geq$99% (Fig. 4.8). Furthermore, the endotoxin concentrations were below the detection level of the assay (Lumilus lysate assay) for all RhIL-3 preparations obtained by this purification protocol. Biological characterization of homogenous RhIL-3 preparations will be presented in the next chapter.

As alluded to earlier, antibodies directed against the hIL-3 polypeptide are able to detect the RhIL-3 protein as well. Polyclonal rabbit anti-human IL-3 antiserum appeared to be approximately 2-3 fold less effective in detecting RhIL-3 in comparison to hIL-3, which is most likely caused by differences in the antigenic determinants between these IL-3 species. Furthermore, analysis of ascites-derived anti-human IL-3 monoclonal antibodies demonstrated that only the monoclonals designated as MCA/A4 and MCA/A8 (Dorssers et al., 1991) react with the RhIL-3 polypeptide (Fig 4.9).

Analysis of interactions between deletion mutants of hIL-3 (Dorssers and Van Leen, 1991) and protein-A purified MCAs, either derived from supernatant of hybridoma cultures or ascites fluids, revealed that the majority of the MCAs are directed against epitopes located between residues 30 and 50 of the mature hIL-3 (Dorssers and Van Leen, 1991; Dorssers et al., 1991). The availability of homogenous RhIL-3 provided a number of amino acid substitutions which facilitated a further and more detailed antigenic epitope determination of hIL-3. In particular, the five ascites-derived monoclonals (Dorssers et al., 1991) were used to map antigenic determinants resulting in the identification of three continuous epitopes and as yet one unidentified epitope. The ascites-derived MCA/A1 and MCA/A18 were shown to react with a linear polypeptide chain located between amino acid 30 to 35 of the mature hIL-3 and MCA/A8 was shown to be directed against the continuous epitope located between residue 40 and 45. The third identified epitope was located, adjacent to the previous 40-45 determinant, between residue 45 and 50 and reacts with MCA/A5. MCA/A4 appeared to react with all constructed deletion mutants as well as the RhIL-3

polypeptide. The epitope specificity of the latter MCA remains to be elucidated. Since MCA/A8 detects RhIL-3, the exchange of a glycine residue by a glutamine at position 42 (Fig. 4.6) apparently does not influence the antigenicity of the 40-45 determinant. In contrast, amino acid alterations at position 34 and 35 (Fig. 4.6) in the 30-35 determinant as well as the alterations at position 46 and 49 (Fig. 4.6) in the 45-50 determinant were shown to abolish the antibody/ligand interaction.

4.6 Summary and Discussion

The gene encoding rhesus monkey interleukin-3 (RhIL-3) was isolated by screening a Lambda Charon 40 library containing rhesus monkey genomic DNA with human interleukin-3 (hIL-3) cDNA. The nucleotide sequence of the RhIL-3 gene displays 92.9% homology with the corresponding part of the hIL-3 gene. Proposed regulatory elements, present in the RhIL-3 gene, are similar to those identified in IL-3 genes of other species, including mouse, rat and man, indicating a common mechanism for the regulation of IL-3 gene expression, with an essential role for these structural elements. Furthermore, the presence of significant homologies in the 5'-flanking regions of several HGF genes such as IL-3, GM-CSF, G-CSF, IFN-γ, IL-2 and its receptor (Kaushansky, 1987) suggests a functional correlation. Most of these HGFs are produced by T lymphocytes in response to inflammatory mediators (Ihle et al., 1989). In addition, the GM-CSF and IL-3 genes are localized, both in mouse and man, in close proximity on the same chromosome. Based on these observations, it has been suggested that GM-CSF and IL-3 and perhaps other HGFs are coordinately released by T lymphocytes (Barlow et al., 1987) and that the well-conserved "decanucleotide" (Stanley et al., 1985) is the responsive element that accounts for the coordinate expression of these HGF genes. However, this element was also identified in the genes encoding human and murine G-CSF which is a factor that is not produced by activated T cells. Thus, the "decanucleotide" is apparently not the only responsive motif involved in the coordinate expression of HGFs by T lymphocytes. A second cytokine-specific sequence (CK-2), located as shown in Fig. 4.5 at the 3' end of the previously identified cytokine-specific "decanucleotide" (CK-1), was identified in the genes encoding GM-CSF and IL-3, but not in G-CSF or IL-2 (Shannon et al., 1988). CK-2 (consensus "TCAGPuTA") was found to be conserved in mouse, human and rhesus monkey IL-3. Since it has been demonstrated that a region spanning CK-1 and CK-2 is specifically recognized by phorbol 12-myristate 13-acetate (PMA) induced, T cell-derived nuclear proteins, it has been hypothesized that binding of these nuclear factors to the responsive elements CK-1 and CK-2 accounts for the coordinate expression of GM-CSF and IL-3 in activated T cells. However, it was shown by Mathey-Prevot et al. (1990) that the target DNA sequences for IL-3 regulation are contained within a 315 nt region upstream of the mRNA start

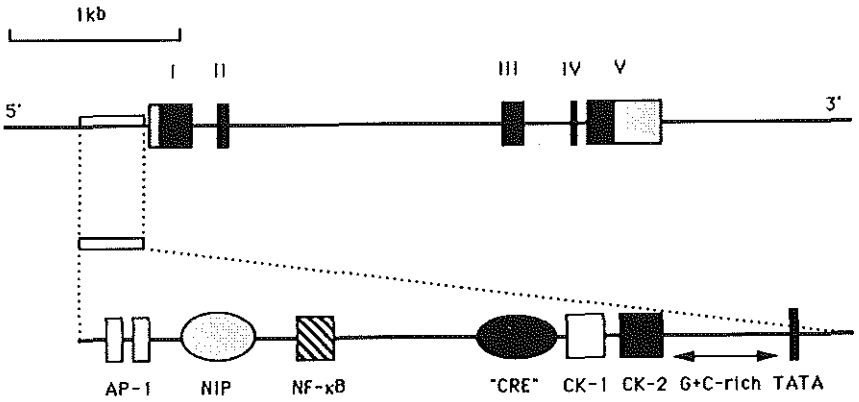


Fig. 4.10 Schematic representation of the putative promoter region of the IL-3 gene. The genomic organization of the IL-3 gene, based on the human (Yang et al., 1987) and rhesus monkey IL-3 (Burger et al., 1990b) sequences is shown at the top. The black boxes at the top figure represent exon I to V. The corresponding noncoding regions of exon I and V are indicated in grey. The promoter region of the IL-3 gene is shown at the bottom. The various structural elements present in the 5'-flanking region of the IL-3 gene are discussed in section 4.2 and 4.6 of this chapter.

site. These investigators demonstrated that CK-1 and CK-2 do not play a role in T cell expression of IL-3. Instead, the sequence "TTACGTCT" also present in rhesus monkey (Fig. 4.4 pos. 1089) and closely related to the established cyclic-AMP-responsive element CRE/ATF (Montminy et al., 1986) was shown to be involved. This "CRE"-like motif, together with nucleotides immediately upstream of this motif appeared to be a prerequisite for efficient expression in activated T cells. In addition, Mathey-Prevot et al. (1990) demonstrated that the presence of a more distally located sequence encompassing an AP-1 binding site (located at an relative identical position in RhIL-3 as described in section 4.2) enhances IL-3 expression. A specific DNA binding site, called NIP (GCTGCCATG) and conserved in rhesus monkey (Fig 4.4 pos. 977), is located between the two binding sites for positive regulators ("CRE" and AP-1). The latter motif (NIP) appeared to function as a potent repressor in the absence of the AP-1 site (Mathey-Prevot et al., 1990). Whether the NF-κB site (section 4.2) and the more distally located copies of the CTF/NF-1 motif indeed affect the expression of IL-3 remains to be elucidated. The NF-κB transcriptional element is common to the promoter region of many cytokine genes, including the promoters of IL-2 and its receptor, IL-6, TNF-α, GM-CSF, G-CSF and IFN-β and was also found in HTLV-I and Human immunodeficiency virus (HIV) suggesting that this transcription factor is involved in the activation of these genes in T cells (Schreck and Baeuerle, 1990; Nishizawa and Nagata,

1990; Libermann and Baltimore, 1990). A schematic representation of the putative promoter region and the intron/exon structure of the IL-3 gene (based on the human and rhesus monkey sequences) is depicted in Fig. 4.10.

The protein encoded by the RhIL-3 gene, which appeared to be 9 residues shorter than the human protein, displays a homology of 80.5% for the mature protein. The two cysteine residues are conserved, whereas only one potential N-linked glycosylation site is conserved. Comparison of the human and rhesus monkey IL-3 coding sequences revealed that the majority of substitutions has occurred at amino acid replacement sites implicating a rapid evolution of the IL-3 protein. This aspect of IL-3 will be discussed in detail in chapter 6.

Large-scale production of RhIL-3 has been accomplished by expression of a RhIL-3 cDNA. This cDNA was constructed from mRNA of COS cells transfected with a genomic fragment encompassing all RhIL-3 coding sequences. A simple purification procedure to obtain preparations of homogenous and endotoxin free *B. licheniformis*-derived RhIL-3 is described in this chapter. The availability of such RhIL-3 preparation enables *in vitro* characterization of the stimulatory activity of RhIL-3, including the capacity to support colony formation of rhesus monkey progenitors, the capacity to support proliferation of human IL-3 dependent AML-193 cells and of normal human bone marrow progenitors. These biological analyses and the concomitant assessment of the specific activity of IL-3 will be presented in the next chapter.

The validity of the hypothesis, as presented in the introduction of this chapter, that a species specificity of hIL-3 accounts for the limited *in vivo* effects of hIL-3 observed in *Macaca* species, can be tested *in vitro* by assessment of the cross-reactivity of RhIL-3 as well as hIL-3 on human and rhesus monkey hemopoietic cells. Furthermore, preparations of homogenous and endotoxin free RhIL-3 facilitate preclinical *in vivo* studies on homologous IL-3 in *Macaca* species. Those *in vivo* studies on homologous RhIL-3 in rhesus monkey are likely to be less hampered by immunological reactions directed against the exogenous IL-3 due to the absence of species barriers. Since *in vivo* HGFs can act in concert, i.e., be additive, synergistic, inhibitory, or in a hierarchical order, it will be essential to study the interacting relationships between IL-3 and other cytokines by administration of homologous IL-3 in combination with other more pathway restricted HGFs in non human primates. Such preclinical studies are facilitated by the availability of recombinant HGFs and are indispensable to define the potential therapeutic role of IL-3.

The molecular cloning of RhIL-3 promoted studies on the identification of antigenic determinants in hIL-3 polypeptide, as described in this chapter. Construction of hIL-3 mutants with individual amino acid substitutions based on the primary structure of the RhIL-3 may further help to identify novel residues involved in the antigenic interactions. The latter may be of interest for the construction of "second generation" hIL-3 proteins with modulated (increased or decreased) antigenicity or biological activity.

CHAPTER 5

BIOLOGICAL CHARACTERIZATION OF RECOMBINANT HUMAN AND RHESUS MONKEY INTERLEUKIN-3

Parts of this chapter have been published in:

*Dorssers, L.C.J., H.Burger, F.J.Bot, R. Delwel, A.H.M. Geurts van Kessel, B. Löwenberg and G. Wagemaker. Characterization of a human multilineage-colony-stimulating factor cDNA clone identified by a conserved noncoding sequence in mouse interleukin-3. Gene **55**: 115-124, 1987.*

*Burger, H., R.W. van Leen, L.C.J. Dorssers, N.L.M. Persoon, P.J. Lemson and G. Wagemaker. Species specificity of human interleukin-3 demonstrated by cloning and expression of the homologous Rhesus monkey (Macaca Mulatta) gene. Blood. **76**: 2229-2234, 1990.*

*Wagemaker, G., F.C.J.M. van Gils, H. Burger, L.C.J. Dorssers, R.W. van Leen, N.L.M. Persoon, J.J. Wielenga, J.L. Heeney and E. Knol. Highly increased production of bone marrow derived blood cells by administration of homologous interleukin-3 to Rhesus monkeys. Blood. **76**: 2235-2241, 1990.*

*Wagemaker, G., H. Burger, F.C.J.M. van Gils, R.W. van Leen and J.J. Wielenga. Interleukin-3. Biotherapy. **2**: 337-345, 1990.*

*Van Leen , R.W., J.G. Bakhuis, R.F.W.C. van Beckhoven, H. Burger, L.C.J. Dorssers, R.W.J. Hommes, P.J. Lemson, B. Noordam, N.L.M. Persoon and G. Wagemaker. Production of human interleukin-3 using industrial microorganisms. Biotechnology. **9**: 47-52, 1991.*

5.1 Introduction

Isolation of a human IL-3 cDNA, identified by exploiting the rather high degree of sequence homology in the 3' noncoding region of the mouse multilineage-colony-stimulating factor (multi-CSF) or interleukin-3 (IL-3) cDNA, has been discussed in chapter 3. Molecular characterization demonstrated that the novel human sequences are genetically related to murine and rat IL-3 (see chapter 3). Characterization of RhIL-3 was enabled by the isolation and expression of the genomic sequences encompassing all RhIL-3 coding sequences and construction, isolation and expression of a RhIL-3 cDNA (see chapter 4). Analogous to the murine system (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966, the development of *in vitro* assays for the clonal growth of human hemopoietic progenitor cells (Pike and Robinson, 1970) allowed for the characterization of soluble human factors capable of interacting directly with PHSCs and committed progenitors to stimulate their proliferation and differentiation.

Biological data presented in this chapter will demonstrate that the isolated human cDNA, genetically related to rodent IL-3, is also functionally related to mouse and rat IL-3. Furthermore, biological characterization of rhesus monkey IL-3 (RhIL-3) will be presented. The production of RhIL-3 was initiated to perform studies on homologous IL-3 in nonhuman primates such as the rhesus monkey. Furthermore, indications towards a species specificity of hIL-3 were observed as described in chapter 4. Therefore, comparisons between the stimulatory activity of hIL-3 and RhIL-3 on human as well as rhesus monkey hemopoietic cells were performed in order to assess the cross-reactivities of these HGFs.

5.2 Biological characterization of human interleukin-3

5.2.1 COS[pLB4] conditioned medium

At the time of the biological characterization of supernatant from COS cells transfected with pLB4, a plasmid carrying the isolated D11 cDNA (Dorssers et al., 1987; chapter 3), neither a human IL-3 dependent cell line nor antibodies directed against the human IL-3 polypeptide were available. Therefore, the COS[pLB4] conditioned medium (CM) was tested for its capacity to support colony formation by human bone marrow cells and to stimulate the proliferation of human acute myelogenous leukemia (AML) blasts. The CM of COS cells transfected with the vector without insert (pL0) was used as control COS medium. Fig. 5.1 shows that COS[pLB4] CM efficiently stimulated *in vitro* colony formation by human hemopoietic progenitors depleted of myelomonocytic and T-lymphocytic accessory cells, whereas COS[pL0] CM failed to stimulate colony formation (not shown). Furthermore, the data presented in Fig. 5.1 demonstrate that the protein encoded by the human cDNA

clone D11 stimulates human bone marrow progenitors committed to several hemopoietic differentiation lineages such as erythroid, myeloid and multipotent progenitor cells. These results demonstrate that the novel factor functions as a multilineage hemopoietic growth factor. Surprisingly, stimulation of rhesus monkey bone marrow cells by COS[pLB4] CM yielded no significant response (see section 5.4) suggesting a species specificity of this factor. Due to the low expression level in COS cells, a detailed analysis of the differential response of COS cell derived hIL-3 on human and rhesus monkey bone marrow could not be pursued.

The responses of purified AML blasts of five patients to COS[pLB4] CM were examined in proliferation and clonal assays as described in detail in chapter 2. Briefly, the clonal assay permits assessment of the colony-stimulating activity of the COS[pLB4] CM by determining its capacity to support colony formation by purified AML blast cells. For the proliferation assays AML blast cells were stimulated with graded percentages (vol/vol) of COS[pLB4] CM and induction of DNA synthesis was assessed by the incorporation of radioactive thymidine ([^{3}H]TdR). AML blast cells of three of the five patients studied proliferated in both assays in response to COS[pLB4] CM. Representative dose-response relationships for colony formation and DNA synthesis of AML blast cells obtained from different patients are shown in Fig. 5.2. CM of mock transfected COS cells did not affect the proliferation of the AML blast cells in both assays. The differential responses of leukemic cells of individual patients to M-CSF, GM-CSF, G-CSF or COS[pLB4] CM, as described elsewhere, (Delwel et al., 1987) demonstrate that the novel factor present in COS[pLB4] CM is distinct from M-CSF, GM-CSF and G-CSF. The variable pattern of responses of AML precursors to the novel factor and the other CSFs studied (Delwel et al., 1987/1988) enabled further phenotypic identification and classification among the leukemias of different patients.

The results shown in Fig. 5.1 and 5.2 demonstrate that the D11 cDNA clone contains the genetic information for a biologically active protein. In addition, the presented data reveal that the encoded protein is excreted following expression in COS cells. Furthermore, the biological actions exerted by the COS produced protein on human hemopoietic cells presented in Fig. 5.1 and 5.2 are analogous to those observed when mouse hemopoietic cells were stimulated by murine IL-3 (Ihle et al., 1983; Rennick et al., 1985). Thus, this human novel factor resembles murine IL-3 with respect to its biological function as well as its biochemical and genetic features such as similar structure of the genes and conservation of gene localization (see chapter 3). Based on these observations, it was concluded that the characterized protein represents the human homolog of murine interleukin-3 (hIL-3). Hence, the protein encoded by the cDNA clone D11 is designated hIL-3 although occasionally, in view of its major biological effects, the term human multilineage-colony-stimulating factor (hmulti-CSF) has been used.

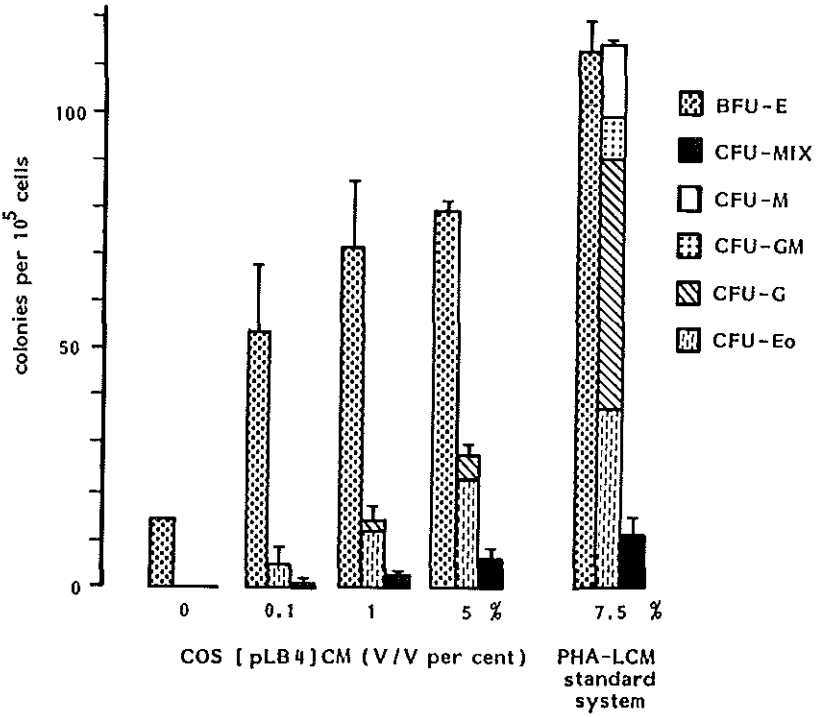


Fig. 5.1 Biological activity of COS[pLB4] CM on colony formation of normal human hemopoietic progenitor cells. Human hemopoietic bone marrow cells, depleted of myelomonocytic accessory cells and T-lymphocytes, were prepared and cultured as detailed in section 2.2.4 of chapter 2. Colonies were classified as CFU-M, CFU-GM, CFU-G, CFU-Eo, BFU-E or CFU-MIX (containing at least erythroid plus myeloid cells) according to standard criteria (60% of the colonies were picked and identified by microscopical analysis). Colony numbers (±SD) were calculated per 10^5 original (unfractionated) nucleated BM cells. The percent COS[pLB4] CM added to the cultures is indicated. In parallel experiments 7.5% PHA-LCM, as a source of HGFs, was added. Mock transfected CM failed to stimulate colony formation (not shown).

As a consequence of the moderate expression level using the COS cell system, the concentration of recombinant hIL-3 in the COS[pLB4] CM is rather low which is reflected in the high concentration of CM required for maximal stimulation of AML blast cells (Fig. 5.2) and human hemopoietic bone marrow cells (Fig. 5.3). The number of IL-3 units, determined in several preparations of CM of independently transfected COS cells, varied from 10^4 to 10^5 U/l. Notably, an unit was arbitrarily defined as the amount of IL-3 required for half-maximal proliferative response in the DNA synthesis assay, using AML blast cells.

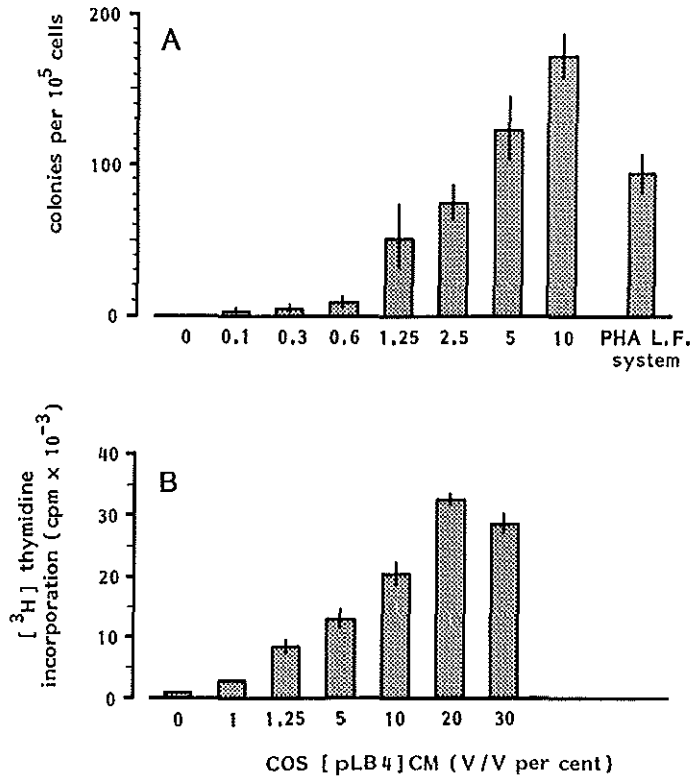


Fig. 5.2 Induction of proliferation of purified AML blast cells by COS[pLB4] CM as determined by a colony formation assay (panel A) and by the DNA synthesis assay (panel B). AML blast cells of two patients were obtained and purified as described in chapter 2. The colony formation assay (patient 1) and DNA synthesis assay (patient 2), as measured by [^{3}H] thymidine incorporation, were performed as detailed in chapter 2. Colony numbers (±SD) were calculated per 10^5 cells plated. The percent COS[pLB4] CM added to the cultures is indicated. In parallel experiments 7.5% PHA-LCM, as a source of HGFs, was added. Addition of mock transfected CM did not affect proliferation of AML blast cells in both assays (not shown).

The variations are likely to be due to different response characteristics of purified AML blast prepared from different patients as well as differential transfection efficiencies. Whether the low expression level originated from selective IL-3 mRNA degradation mediated by the AU-rich sequences present in the hIL-3 cDNA (see chapter 3; Shaw and Kamen, 1986; Kruys et al., 1989;

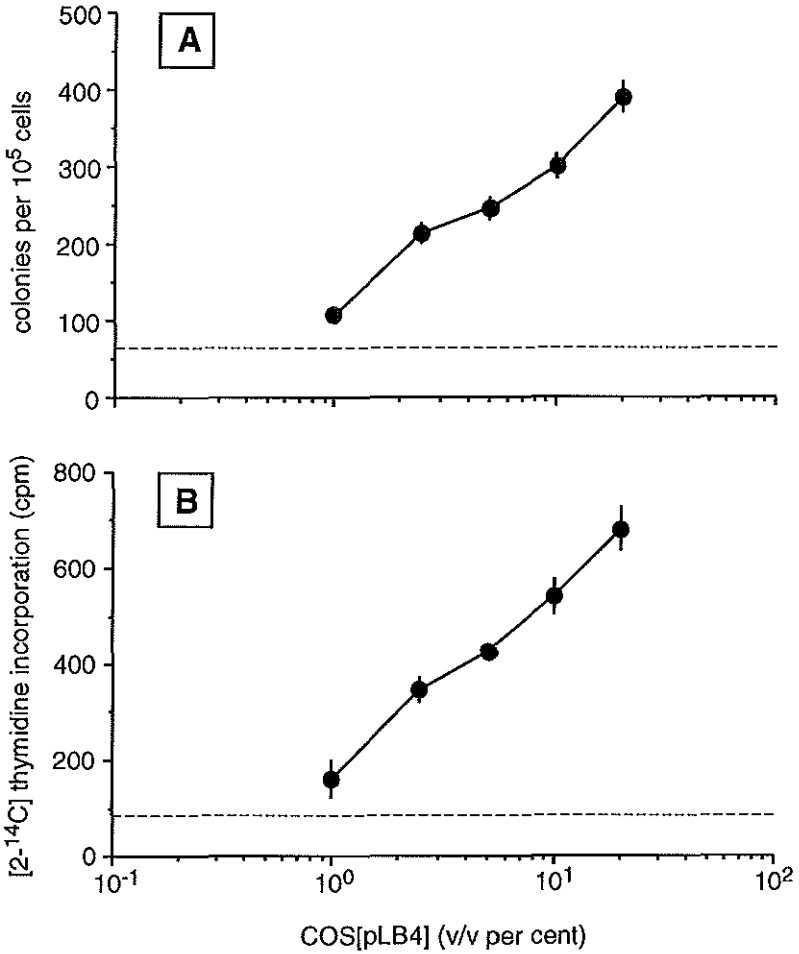
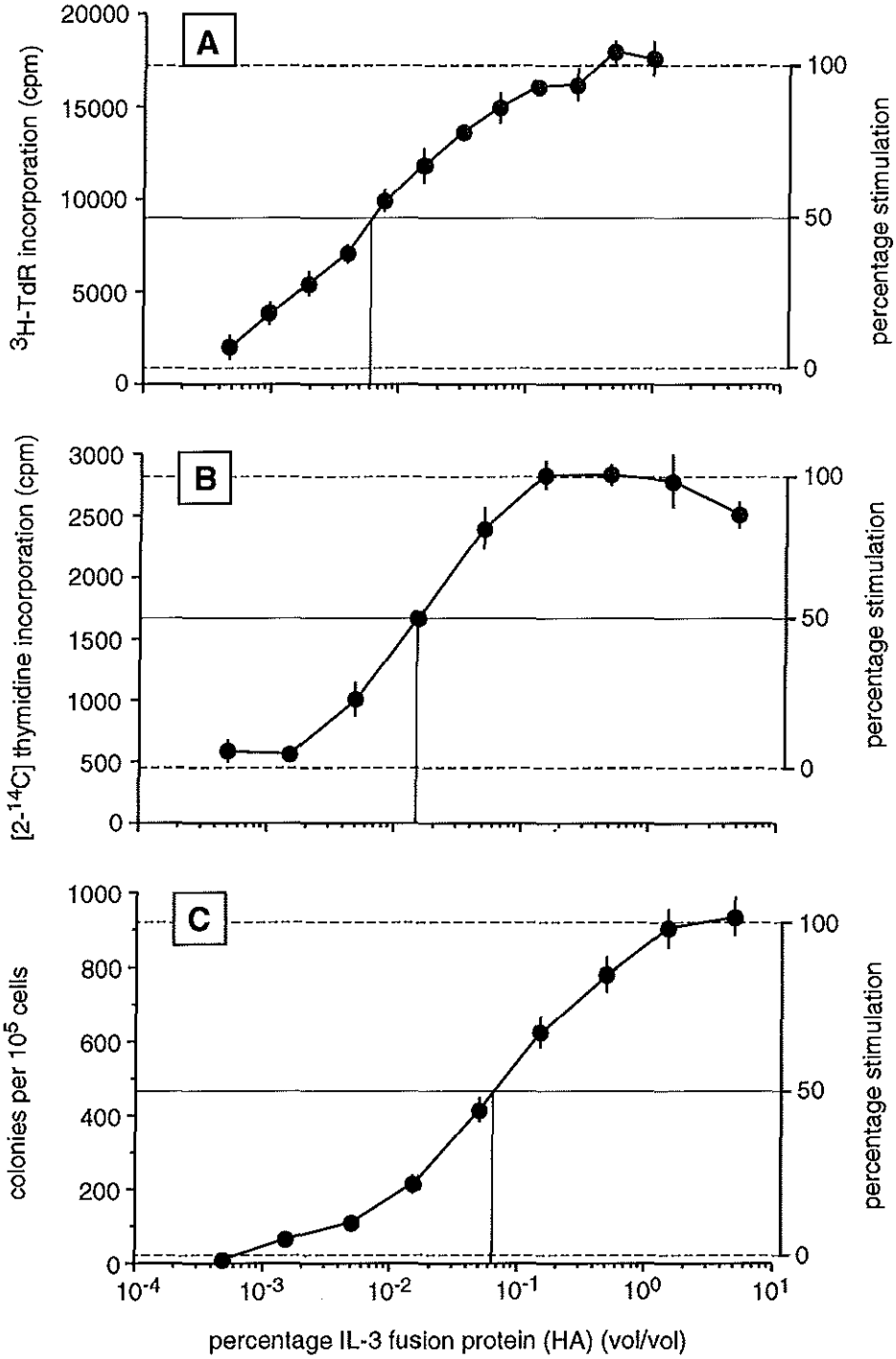


Fig. 5.3 Effect of COS[pLB4] CM on colony formation (panel A) and DNA synthesis (panel B) by human bone marrow progenitor cells. Human bone marrow progenitor cells were prepared and cultured as detailed in chapter 2. Colony numbers (±SD) were calculated per 10^5 original (unfractionated) nucleated BM cells. The percent COS[pLB4] CM added to the cultures is indicated. The DNA synthesis assay (panel B), as measured by [^{3}H] thymidine incorporation, was performed as detailed in chapter 2. Addition of mock transfected CM did not affect the proliferation of hemopoietic bone marrow cells in both assays (not shown).

Malter, 1989) or was associated with the kind of eukaryotic expression system was investigated by analyzing COS[pLH1] CM. Plasmid pLH1 was derived from pLB4 (Fig. 3.11 and 3.12) by deletion of the 3' noncoding sequences between the *Ava*I site (pos. 541; Fig. 3.5 and 3.8) and the *Xho*I site (pos. 856; Fig. 3.5 and 3.8) which encompass the "ATTTA" repeat units. Comparison between COS[pLB4] and COS[pLH1] CM revealed that the number of IL-3 units did not


A
B
C
^{3}H-TdR incorporation (cpm)
[2-^{14}C] thymidine incorporation (cpm)
colonies per 10^5 cells
percentage stimulation
percentage IL-3 fusion protein (HA) (vol/vol)
20000
15000
10000
5000
0
3000
2500
2000
1500
1000
500
1000
800
600
400
200
100
50
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}

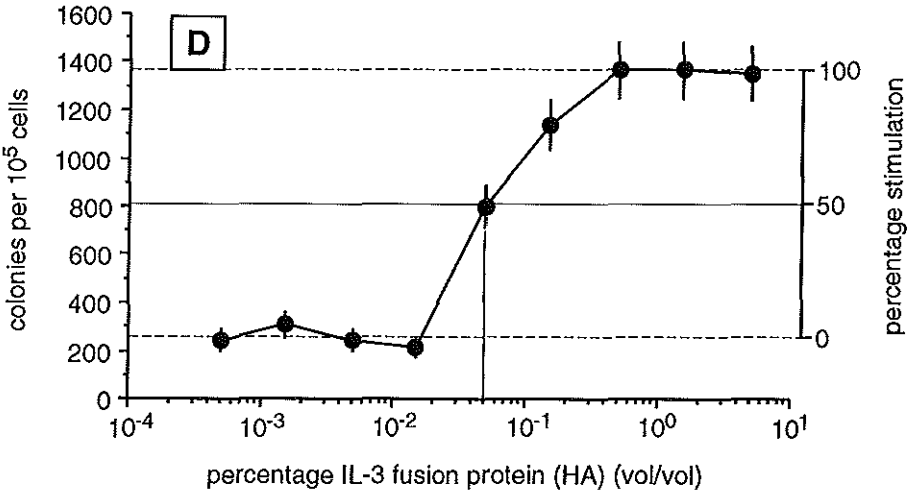


Fig. 5.4 Effect of partially purified bacterial hIL-3 fusion protein on AML blast cells (panel A) and human bone marrow (panel B-D). The AML proliferation assay and the various human bone marrow assays, i.e., human BM proliferation assay (panel B), human BM colony formation assay (panel C), and the human BM liquid culture assay (panel D) are described in section 2.2.4 of chapter 2. The percentage of preparation "HA" (hIL-3 fusion protein derived from pUC/hMultiΔ1) required for half maximal stimulation is indicated for the various assays.

differ significantly indicating that IL-3 expression in COS cells is not markedly influenced by these sequences. Purification of COS cell-derived hIL-3 and assessment of the specific activity was not done due to the rather low expression level.

5.2.2 LacZ/hIL-3 fusion proteins

The construction of lacZ/hIL-3 bacterial expression vectors and subsequent production of IL-3 fusion proteins was initiated to enable the production of specific antibodies directed against the hIL-3 polypeptide (see chapter 3) but concomitantly facilitated biological characterization of the lacZ/hIL-3 fusion proteins. Partially purified hIL-3 fusion protein preparations were assayed for the presence of biological activity on human hemopoietic cells.

The initial assays used to determine the biological activity of the hIL-3 fusion proteins included assessment of the capacity to activate DNA synthesis of purified AML blast cells which will be referred to as the "AML proliferation assay", induction of DNA synthesis of human bone marrow progenitor cells which will be referred to as the "hBM proliferation assay", capability to support colony formation by human bone marrow progenitor cells which will be

referred to as the "hBM colony formation assay" and an assay, referred to as the "hBM liquid culture assay". The latter assay measured the production of GM-CSF responsive progenitor cells, determined by the development of colonies in the presence of GM-CSF as a consequence of stimulation of human bone progenitors in liquid cultures by IL-3 (see chapter 2, section 2.2.4).

A particular hIL-3 fusion protein preparation was analyzed using these various assays. The analyzed hIL-3 fusion protein was expressed by *E. coli* following transformation of the bacteria with construct pUC/hIL-3Δ1 (see chapter 2 and 3). The unglycosylated hIL-3 fusion protein consisting of the first 11 aa of lacZ fused to aa 2 to 133 of the mature hIL-3 polypeptide was partially purified and the resulting hIL-3 fusion protein preparation was designated as preparation "HA". The total protein concentration of preparation "HA" was 0.1 μg per ml and the content of hIL-3 fusion protein, estimated on SDS-polyacrylamide gel (Fig. 3.13; lane 3), was 20%. Representative dose-effect relationships are shown in Fig 5.4A-D. The data indicate that an unglycosylated hIL-3 fusion protein expressed by *E. coli* can be obtained in a biologically active form, at least *in vitro*.

The percentage of preparation "HA" (vol/vol) required for half maximal stimulation was determined for each assay and used to calculate the number of IL-3 units. The percentage required in the AML proliferation assay is 6×10^{-3}% (Fig. 5.4A), the hBM proliferation assay required 1.5×10^{-2}% (Fig. 5.4B), the hBM colony formation assay required 6×10^{-2}% (Fig. 5.4C) and the hBM liquid culture assay required 5×10^{-2}% (Fig. 5.4D) for half maximal stimulation. Thus, the units of IL-3 activity, calculated per ml of preparation "HA", varied from 1.7×10^3 to 17×10^3 depending on the biological assay used (Fig 5.4A-D). Table 5.1 demonstrates that even within a particular assay considerable variations are present indicating that assessment of specific activity and comparison of the biological activity of different IL-3 preparations has to be performed with an internal IL-3 standard. The variations within the AML proliferation assay are quite acceptable but larger variations were observed in the human bone marrow assays (Table 5.1), which are likely attributable to differences of the bone marrow preparations. These differences may be variations of bone marrow constituents of individual donors, fresh or cryopreserved bone marrow preparations, extent of purification of the progenitor cell population and contamination of the final progenitor cell population with accessory cells. The accessory cells have been shown to affect the colony formation of human bone marrow progenitor cells by hIL-3 in a cell number dependent manner (Bot et al., 1988).

The biological activity of various hIL-3 fusion proteins, constructed as described in chapter 3 and schematically depicted in Fig. 3.12, was assessed on AML blast cells using the AML proliferation assay. Large variations within the AML proliferation assay were in part attributable to different IL-3 response characteristics of purified AML blast cells prepared from different patients

Table 5.1 Assessment of biological activity of a partially purified hIL-3 fusion protein preparation designated "HA"

Experiment	Assay type[a]			
	AML proliferation	hBM proliferation	hBM colony formation	hBM liquid culture
	IL-3 activity expressed as units (U) per ml[b]			
1[c]	17	7	2	2
2	12	3	3	10
3	13	50	5	ND[d]
4	10	50	3	ND
5	20	ND	ND	ND

[a] The different assays are described in this section (5.2.2) and chapter 2. Determination of the dilutions required for half maximal stimulation in the various assays was assessed analogous to those shown in Fig. 5.3A-D.
[b] IL-3 activity is expressed as U x 10^{-3} and calculated per ml. The amount of IL-3 per ml, required to give half-maximal response is designated as 1 unit (see also chapter 2 section 2.2.2).
[c] Numbers shown for this experiment are derived from dose-effect relationships shown in Fig. 5.3A-D.
[d] Not determined.

Table 5.2 Assessment of specific activity of various bacterial hIL-3 fusion proteins by the AML proliferation assay.

bacterial fusion protein construct	molecular weight (kDa)	production level[a]		specific[b] activity (kU/mg)
		protein(mg/l)	activity (U/l)	
pUC/hMulti	20	20	0.01	5
pUC/hMultiΔ1	16	5	2.5	500
pUC/hMultiΔ2	15	1	0.5	500
pUR/hMulti6	130	80	ND[c]	<0.01

[a] Production of bacterial hIL-3 fusion proteins was calculated per liter of starting culture. Activity is expressed as units x 10^{-6} per liter and hIL-3 protein concentration as mg per liter.
[b] IL-3 concentrations were estimated on SDS-polyacrylamide gel and the activity was determined in the AML proliferation assay. Specific activity is expressed as units x 10^{-3} per mg hIL-3 fusion protein.
[c] Not determined.

(Delwel et al., 1987/1988). To circumvent the problem of considerable variation within a particular IL-3 preparation, cryopreserved preparations of selected and purified AML blast cells of a single patient were used for unit calibration of distinct IL-3 preparations. For each hIL-3 fusion protein preparation the corresponding dose-effect relationship was determined and the number of IL-3 units was calculated from the dilution showing half maximal stimulation. Furthermore, the hIL-3 fusion protein concentrations of partially purified bacterial fusion protein preparations were estimated on SDS-polyacrylamide gels (Fig. 3.13) and used to calculate specific activities.

The activity of various hIL-3 fusion proteins in the AML proliferation assay is shown in Table 5.2. The calculated specific activities (summarized in Table 5.2) demonstrate that the 20 kDa hIL-3 fusion protein encoded by the pUC/hMulti is approximately 100-fold less active than the 15 and 16 kDa hIL-3 fusion proteins encoded by pUC/hMultiΔ2 and pUC/hMultiΔ1, respectively. The diminished activity of the 20 kDa fusion protein may be explained by the presence of the hydrophobic leader peptide which presumably interferes with the desirable protein conformation for optimal activity. The hIL-3 fusion protein lacking 14 N-terminal residues, encoded by pUC/hMultiΔ2, retains its biological activity (Table 5.2). This result indicates that these 14 N-terminal residues of the mature hIL-3 protein are dispensable or their function is replaced by the substituted lacZ-derived amino acids. The 15 and 16 kDa hIL-3 fusion proteins exerted half-maximal stimulation at a concentration of about 2 μg per liter. A comparable construct, carrying only the sequences for the mature hIL-3 immediately downstream of the initiating methionine of lacZ and encoding a 15 kDa mature hIL-3 with the initiating f-methionine still present at the N-terminus of the protein (Van Leen et al., 1991), exerted biological activity similar to the 15 and 16 kDa hIL-3 fusion proteins. Apparently, the heterologous amino terminus of the lacZ/hIL-3 fusion proteins encoded by pUC/hMultiΔ1 or pUC/hMultiΔ2 (Fig. 3.12) does not influence the biological activity. In contrast to these 15 and 16 kDa hIL-3 proteins, the 130 kDa β-galactosidase/hIL-3 fusion protein did not show any biological activity in the range of 0.1 μg to 10 mg per liter. It was an intriguing observation that the decrease in production level in terms of IL-3 protein concentration is associated with the number of residues between the initiating methionine of lacZ and the first residue of the mature hIL-3 protein. Apparently, the β-galactosidase sequences have a beneficial influence on the production level. This conclusion is supported by the relatively low production of the 15 kDa hIL-3 mature protein lacking those lacZ sequences (Van Leen et al., 1991). Whether the lacZ sequences stabilize the transcription or translation process or cause alternative intracellular localization and stabilization awaits further investigation. However, analysis of the low expression of some hIL-3 deletion muteins (Dorssers et al., 1991) suggests that destabilization of transcripts may contribute to low production.

Considerable variation of biological activity of the same IL-3 preparation using distinct bioassays was frequently found. Apparently, the biological activity of a growth factor is defined by its biological assay. Therefore, the availability of specific growth factor-dependent cells, analogous to the murine system, seems to be indispensable for the reproducible determination of the specific activity of hIL-3. Furthermore, such cells would be useful to compare specific activities of recombinant hIL-3 preparations derived from different microorganisms. At the time of the production of yeast derived hIL-3 as described in the next section, immortal human cell lines requiring human GM-CSF or human IL-3 for proliferation were established from patients with childhood acute leukemia (Lange et al., 1987). One of these factor dependent cell lines, the IL-3 dependent acute monocytic leukemia cell line AML-193, represents a novel model to study and assess the biological activity of IL-3. Data obtained from AML-193 cells appeared to be reproducible and therefore this cell line is nowadays routinely used to assess the biological activity of hIL-3 preparations. This assay will be referred to as the "AML-193 assay".

5.2.3 Human IL-3 expressed by *Kluyveromyces lactis*

Isolation of recombinant hIL-3 from the cell free medium of a culture of *Kluyveromyces lactis* (*K. lactis*) resulted in yeast derived hIL-3 preparations, which are free of contaminating proteins. Purified hIL-3 turned out to be heterogeneous with respect to its molecular weight, which is inherent to the nature of glycoproteins. Therefore, the term homogeneous used in this section for *K. lactis* derived hIL-3 refers to hIL-3 free of contaminating non-IL-3-like proteins. The yeast derived hIL-3 products ranged in size from about 15 to 100 kDa, with two predominant glycosylated products around molecular weights of 24 and 28 kDa. A distinct band at about 15 kDa was also observed. The latter product most likely corresponds to the mature unglycosylated IL-3. The 24 and 28 kDa products presumably carry different carbohydrate moieties. In addition, the 24 and 28 kDa proteins can be converted into the 15 kDa protein by endoglycosidase H treatment (Van Leen et al., 1991). The decrease in molecular weight (28 to 15 kDa) as a consequence of endoglycosidase H treatment shows that the carbohydrate content of this 28 kDa yeast-derived hIL-3 is approximately 45 %. However, it should be emphasized that the glycosylation is of the high mannose type and differs considerably from the complex mammalian type of glycosylation (Farquhar, 1985). Analysis of the N-termini of these yeast-derived hIL-3 proteins revealed that the first two aa of the mature human IL-3 (Ala-Pro) were missing, which is probably due to the presence of a dipeptidyl aminopeptidase in the Golgi system of yeast cells (Van Leen et al., 1990; Van Leen et al., 1991).

A characteristic dose-effect relationship of homogeneous hIL-3 produced by *K. lactis* is shown in Fig. 5.5. The concentration required for half-maximal

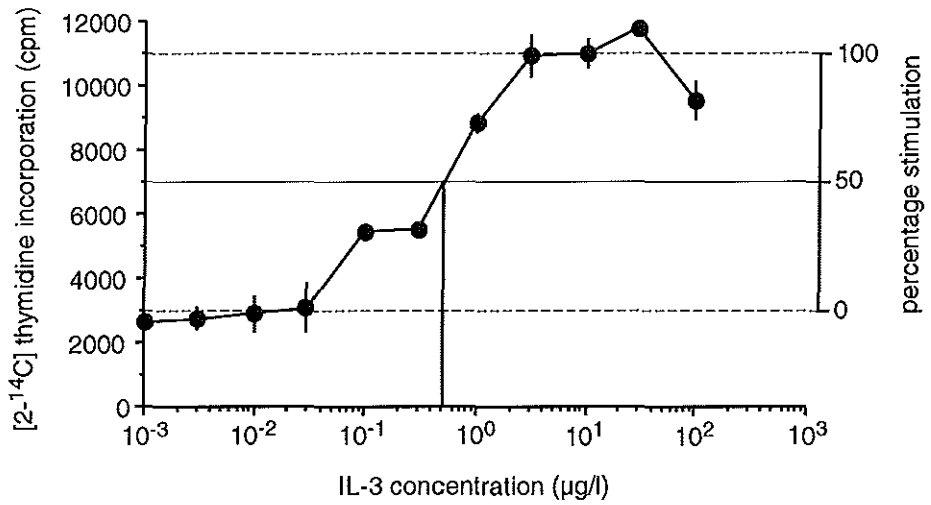


Fig. 5.5 Effect of *K. lactis* derived hIL-3 on human AML-193 cells. The biological activity of yeast derived hIL-3, purified to homogeneity as judged by SDS-PAGE, was determined using the AML-193 proliferation assay (see section 2.2.2 of chapter 2). The concentration of yeast derived hIL-3 required for half maximal stimulation of AML-193 cells is 0.5 µg/l.

stimulation of AML-193 cells varied between 0.5 to 3 µg per liter, which corresponds with 5 x 10^{-11} to 2 x 10^{-10}M for this glycosylated molecule. The effect of *K. lactis* derived hIL-3 on normal hemopoietic cells is shown in Fig. 5.6. The concentrations required to stimulate half-maximal proliferation (Fig. 5.6A) and half-maximal numbers of colonies developed in cultures by human bone marrow progenitors (Fig. 5.6B) are in both assays 3 µg per liter. Thus, the specific activity of yeast derived hIL-3 is comparable to the *E. coli* derived hIL-3. Despite the presence of the yeast type glycosylation and the absence of the first two amino acids of the mature hIL-3, the yeast derived hIL-3 appears to be highly active. A preparation of purified *K. lactis* derived hIL-3 with a specific activity of 1 x 10^6 units per mg protein has been routinely used as an internal hIL-3 activity standard to calibrate IL-3 activities of other preparations.

5.2.4 Human IL-3 expressed by *Bacillus licheniformis*

Purification of recombinant unglycosylated hIL-3, produced and excreted by *Bacillus licheniformis* (*B. licheniformis*), resulted in homogeneous IL-3

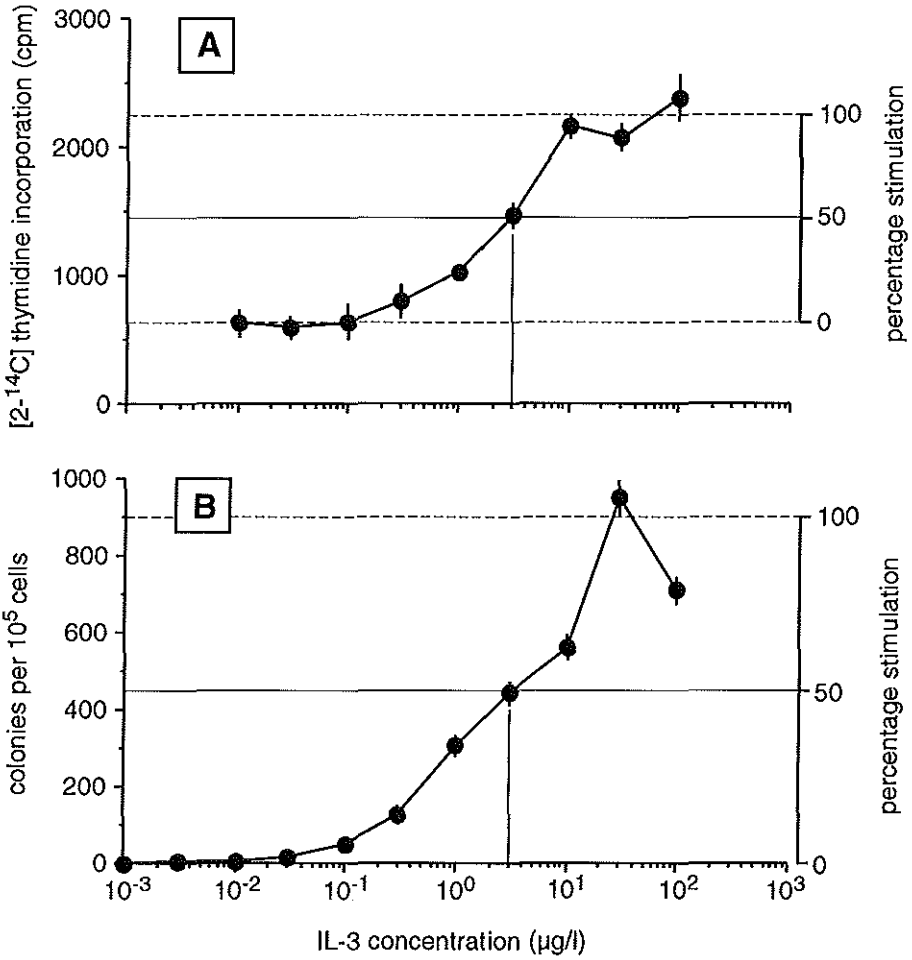


Fig. 5.6 Effect of *K. lactis* derived hIL-3 on DNA synthesis (panel A) and colony formation (panel B) by purified human bone marrow progenitor cells. Homogeneous yeast derived hIL-3 (see also legend of previous figure) was used in these bioassays (see section chapter 2).

preparations, as described in chapter 2. The effect of *B. licheniformis*-derived homogeneous hIL-3 on AML-193 cells is depicted in Fig. 5.7 and the concentration of this particular preparation required for half-maximal response appeared to be 1 µg per liter. Independently produced and purified recombinant *B. licheniformis* derived hIL-3 preparations have been used as internal standards and more than 30 dose-effect relationships were established on AML-

193 cells. The specific activity of these preparations (mean ± standard deviation) was shown to be 1.17 ± 0.77 x 10^6 units per mg protein and ranged from 3 to 30 x 10^5 units per mg protein. Large deviations were always related to an anomaly of the assay such as spontaneous proliferation resulting in high backgrounds and in most cases a lower stimulation over the background. It appears that the AML-193 assay is a reliable method to assess and compare the biological activity of hIL-3 preparations originating from different expression systems.

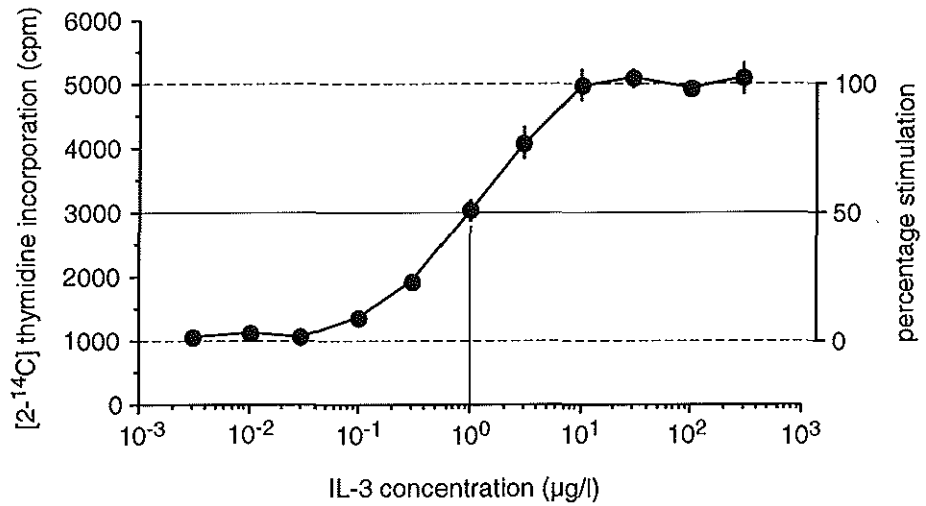


Fig. 5.7 Effect of *B. licheniformis* derived hIL-3 on AML-193 cells. The biological activity of homogeneous *B. licheniformis* derived hIL-3 (purity >95% as judged by SDS-PAGE) was determined using the AML-193 proliferation assay (see section 2.2.2 of chapter 2). The concentration of this bacterial derived hIL-3 required for half maximal stimulation of AML-193 cells is 1.0 µg/l.

Stimulation of human bone marrow by *B. licheniformis* derived hIL-3 showed a dose dependent production of progenitor cell derived colonies (Fig. 5.8) analogous to those observed with hIL-3 fusion proteins (Fig. 5.4C) and *K. lactis* produced homogeneous hIL-3 (Fig. 5.6B). Assessment of the specific activity using the "hBM" assays obviously resulted in larger variations as compared to the AML-193 assay. Based on the results of the different bioassays to determine IL-3 activity, it was concluded that unit calibration and assessment of specific activity of hIL-3 preparations can best be defined by the AML-193 assay.

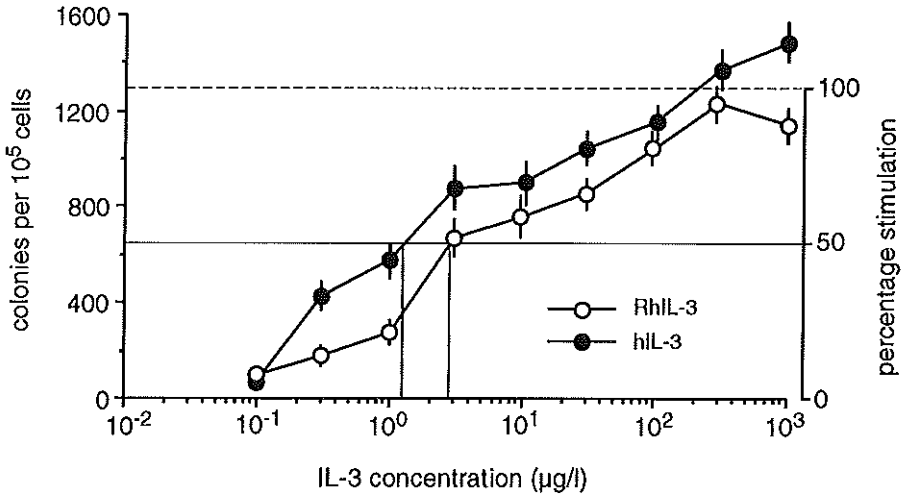


Fig. 5.8 Effect of *B. licheniformis* derived IL-3 on colony formation by human bone marrow progenitor cells. The biological activities of homogeneous *B. licheniformis* derived human IL-3 (hIL-3) and rhesus monkey IL-3 (RhIL-3) were determined using the "hBM colony formation" assay (see section 2.2.4 of chapter 2). The concentration of hIL-3 required for half maximal stimulation of human BM cells was approximately 1.0 µg/l. The purity of both IL-3 preparations was comparable (>95%). Human and RhIL-3 showed comparable stimulatory activity on human BM progenitor cells, demonstrating that RhIL-3 is capable to support the proliferation of human cells (see also section 5.4).

5.3 Biological characterization of rhesus monkey interleukin-3

5.3.1 COS[pSVL/RhIL-3] conditioned medium

The presence of IL-3 related protein in conditioned medium of COS cells transfected with pSVL/RhIL-3 was demonstrated by Western blot analysis using polyclonal and monoclonal antibodies directed against the hIL-3 polypeptide. The presence of functional rhesus monkey IL-3 (RhIL-3) was demonstrated by the capacity of the supernatant to stimulate hemopoietic cells. COS[pSVL/RhIL-3] CM was capable to stimulate proliferation of human AML-193 cells as depicted in Fig. 5.9. However, high concentrations of conditioned medium of mock transfected COS cells (mock CM) appeared to stimulate those cells as well. This phenomenon was not observed with previously produced mock transfected COS cell CM using AML blast cells (see section 5.2.1). Furthermore, the elevated maximal stimulation level observed with COS[pSVL/RhIL-3] CM suggested that, in addition to RhIL-3, other stimuli were present.

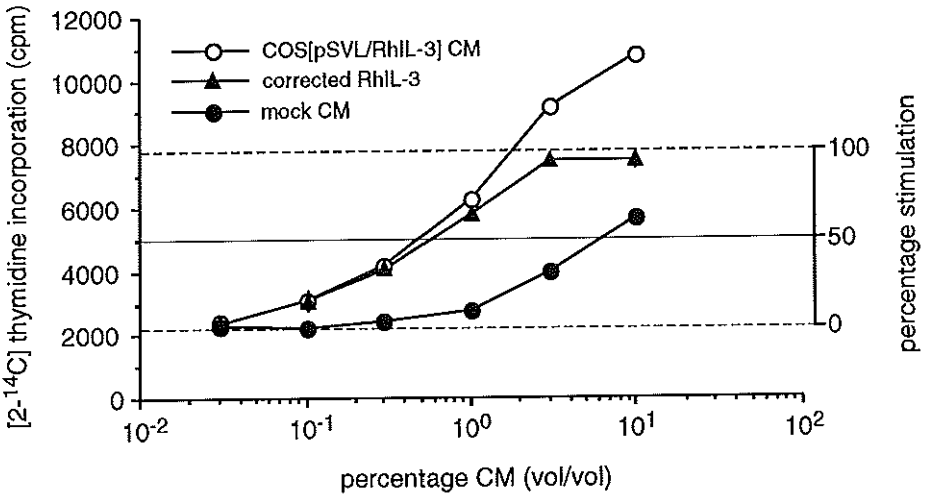


Fig. 5.9 Effect of COS[pSVL/RhIL-3] CM on human AML-193 cells. Stimulatory capacity of COS[pSVL/RhIL-3] CM on human AML-193 cells was measured by [2-^{14}C] thymidine incorporation as described in section 2.2.2 of chapter 2. The effect of CM of COS cells transfected with the empty vector (pSVL) was determined and is indicated as mock CM. The dose-effect relationship indicated by corrected RhIL-3 represents the thymidine incorporation of COS[pSVL/RhIL-3] CM corrected for the observed effect of mock CM by substraction of the additive effect.

The additive effect of mock CM on the dose effect relationships of B. licheniformis-derived hIL-3 (Fig. 5.10A) and recombinant GM-CSF (Fig. 5.10B) demonstrated that the elevated maximal incorporation level had to be attributed to stimulatory activities present in COS cell CM which are apparently not specific enhancers of IL-3 or GM-CSF. In addition to the additive effect of mock CM, Fig. 5.10 shows the stimulatory effects of IL-3 and GM-CSF on AML-193 cells, with GM-CSF being the more potent stimulator. It has been shown that the concentration of the unknown stimuli in CM harvested after 24 and 48 hours was comparable, whereas the RhIL-3 concentration increased substantially. This indicates that the non-IL-3 stimulatory molecules are probably not produced by the transfected cells, but are presumably constituents of the culture medium. Therefore, substraction of the additive effect allows assessment of the number of IL-3 units present in COS[pSVL/RhIL-3] CM. The corrected dose-effect relationship in Fig. 5.9 revealed that 0.55% COS[pSVL/RhIL-3] CM was required for half-maximal response corresponding with about 180 units RhIL-3 per ml of CM. Generally, COS[pSVL/RhIL-3] CM

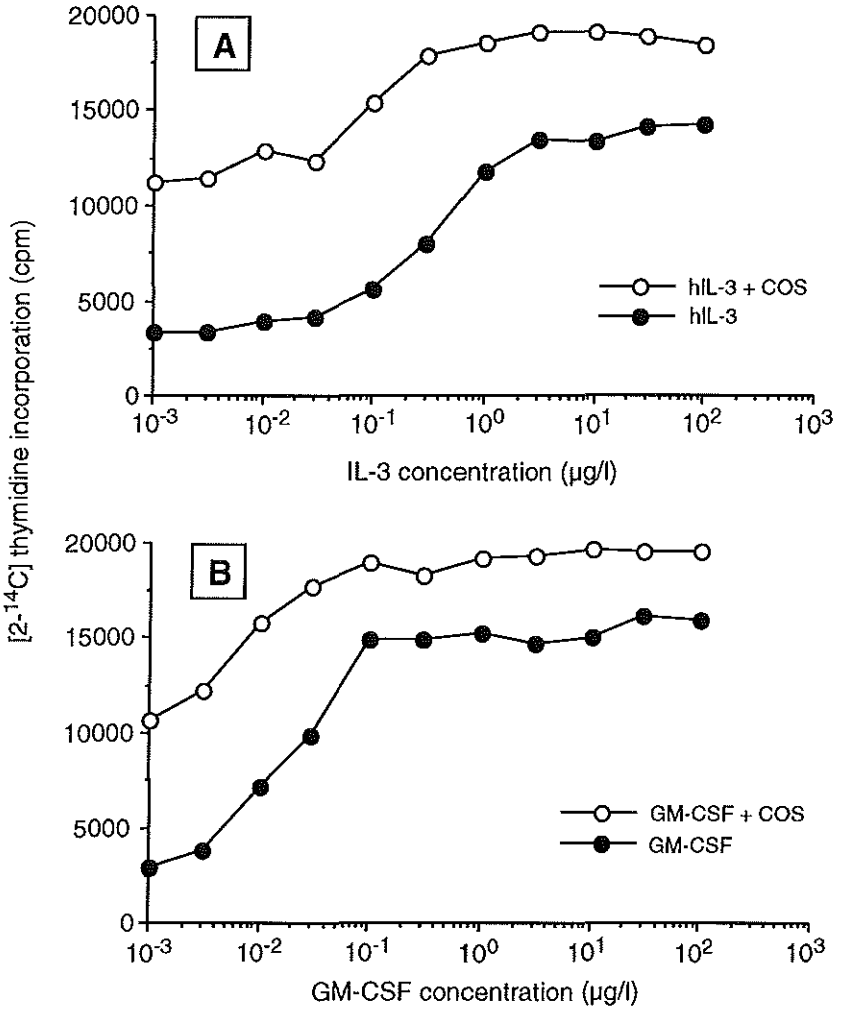


Fig. 5.10 Effect of mock CM on AML-193 cells stimulated by hIL-3 or GM-CSF. The additive effect of 10% mock CM on the dose-effect relationship of *B. licheniformis* derived hIL-3 is shown in panel A, whereas panel B shows the additive effect of 10% mock CM on the dose-effect relationship of recombinant GM-CSF (Glaxo Inc., Genève, Switzerland).

contains somewhat more IL-3 activity than CM of COS cells transfected with hIL-3 cDNA (hIL-3 COS[pLB4] CM). The somewhat higher expression level could be explained either by the different expression vectors or by the presence

of the RhIL-3 intron sequences which most likely stabilize the pre-mRNAs as was suggested by the 5 to 10-fold higher expression in COS cells using the genomic hIL-3 sequences instead of its cDNA (Van Leen et al., 1990; Van Leen et al., 1991).

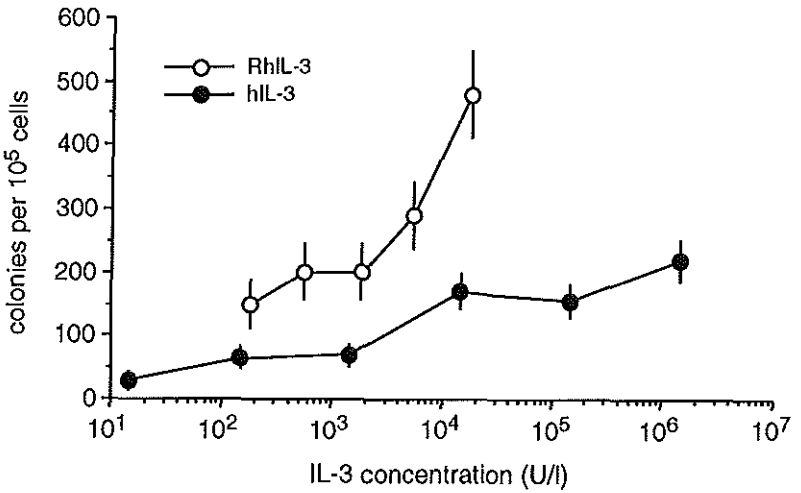


Fig. 5.11 Effect of human and rhesus monkey IL-3 on colony formation by purified rhesus monkey bone marrow progenitor cells. Rhesus monkey BM cells were stimulated either by hIL-3 (purified B. licheniformis derived hIL-3) or by RhIL-3 (COS[pSVL/RhIL-3]. The number of IL-3 units present in these preparations were defined by the AML-193 assay. Mock CM had no stimulatory effect on rhesus monkey BM cells (not shown). The differential effect of human and RhIL-3 on rhesus monkey BM cells is discussed in section 5.4 of this chapter.

In addition to the capacity to stimulate proliferation of human AML-193 cells, COS[pSVL/RhIL-3] CM was capable to support colony formation by rhesus monkey bone marrow progenitor cells as shown in Fig. 5.11. In contrast to AML-193 cells, rhesus monkey bone marrow cells were not stimulated by mock CM (not shown). The biological effects observed with COS cell derived hIL-3 on human bone marrow cells and RhIL-3 on rhesus monkey bone marrow cells (i.e. homologous situations) are comparable. However, differential effects were observed on heterologous bone marrow preparations indicating species specificity, which will be presented and discussed in section 5.4.

5.3.2 Rhesus monkey IL-3 expressed by *Bacillus licheniformis*

Rhesus monkey IL-3 produced by B. licheniformis was subsequently purified to

homogeneity as described in the previous chapter. Stimulatory capacity of independently produced and purified RhIL-3 preparations on AML-193 cells is shown in Fig. 5.12A-C. The dose-effect relationships revealed that 3 µg RhIL-3 per liter is required for half-maximal stimulation. Therefore, the specific activity of RhIL-3, defined by the AML-193 assay, is 3 x 10^5 units per mg protein.

The effects of RhIL-3 on colony formation by rhesus monkey bone marrow cells ("BM colony formation" assay as described in section 2.2.4) is shown in Fig. 5.13A and the capacity to support the production of GM-CSF responsive clonogenic cells in liquid cultures of rhesus bone marrow ("BM liquid culture" assay as described in section 2.2.4) is depicted in Fig. 5.13B. RhIL-3 concentration required for half maximal-response in rhesus monkey bone marrow assays varied considerably depending on the degree of purification of the progenitor cell population. Apparently, assessment of the biological activity of RhIL-3 on rhesus monkey bone marrow is substantially influenced by the content of accessory cells. Formation of colonies in response to RhIL-3 by rhesus monkey progenitor cells was hardly observed when preparations were completely depleted of accessory cells, whereas a high content of accessory cells induced spontaneous colony formation in the absence of any added stimulus. Similar effects were observed in the human situation (Bot et al., 1988).

Results obtained from *in vivo* characterization of RhIL-3, as described elsewhere (Wagemaker et al., 1990b), demonstrate that administration of homologous IL-3 produced by *B. licheniformis* to rhesus monkeys resulted in a dose-dependent stimulation of the production of all bone marrow derived cell lineages (Wagemaker et al., 1990b). In line with the *in vitro* capacity to support colony formation and to stimulate the production of GM-CSF responsive progenitors, as described in this chapter, the *in vivo* results may be simply explained by increased production of progenitor cells derived from early multipotential bone marrow cells in response to IL-3, resulting in augmented production of all bone marrow derived blood cells. The magnitude of IL-3 responses are comparable to those observed after administration of murine recombinant IL-3 to mice (Kindler et al., 1986; Metcalf et al., 1986).

As depicted in Fig. 5.12, the specific activities of independently produced and purified RhIL-3 preparations are found to be comparable in the AML-193 proliferation assay. All RhIL-3 preparations presented in Fig. 5.12 were derived from laboratory-scale fermentations. These homogeneous RhIL-3 preparations exerted half-maximal stimulation of AML-193 cells at 3 µg per liter which corresponds with a concentration of about 2 x 10^{-10} M. In contrast, a RhIL-3 preparation (RhIL-3 IIIB) derived from intermediate-scale fermentation displayed at least 10-fold lower specific activity as determined on AML-193 cells (Fig. 5.14) as well as on human bone marrow progenitor cells (Fig. 5.15). This product, compared to the laboratory-scale derived RhIL-3 protein, did not differ with respect to its protein chemistry. The latter was

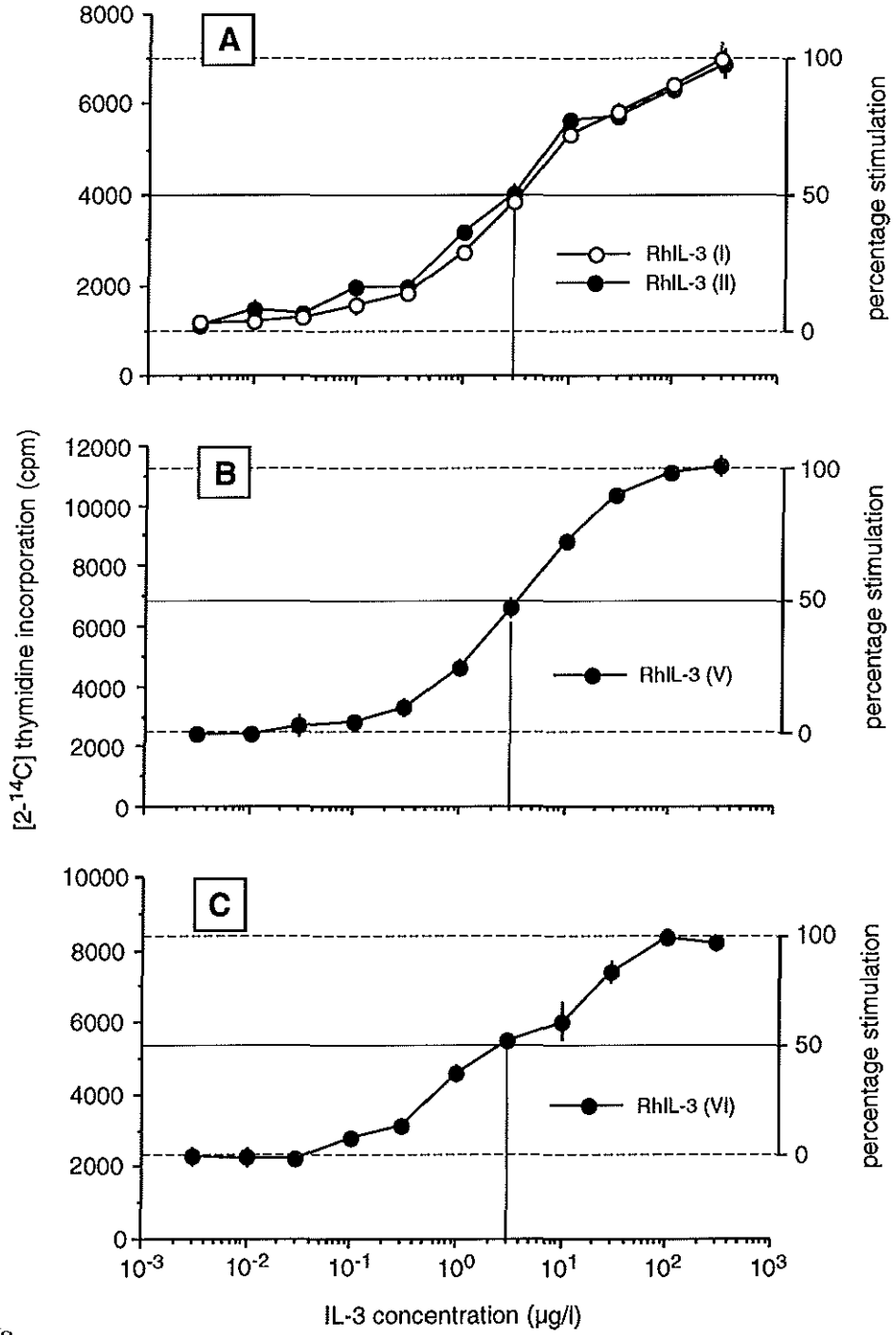

A
B
C
8000
6000
4000
2000
0
12000
10000
100
50
0
RhIL-3 (I)
RhIL-3 (II)
RhIL-3 (V)
RhIL-3 (VI)
percentage stimulation
[2-14C] thymidine incorporation (cpm)
10-3
10-2
10-1
100
101
102
103
IL-3 concentration (µg/l)

Fig. 5.12 Effect of homogeneous *B. licheniformis* derived RhIL-3 on AML-193 cells. Stimulation of DNA synthesis in AML-193 cells by various purified RhIL-3 preparations, i.e., batch I and batch II (panel A), batch V (panel B), and batch VI (panel C) was measured by [2-^{14}C] thymidine incorporation (see section 2.2.2 of chapter 2).

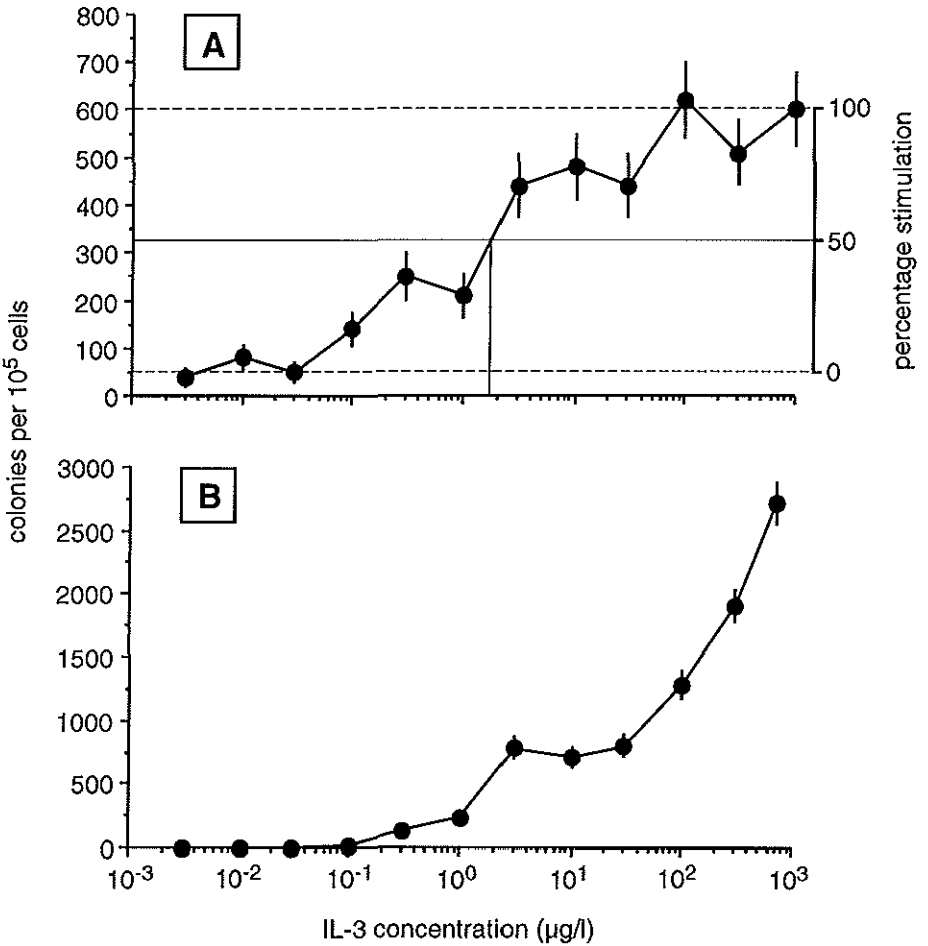


Fig. 5.13 Effect of RhIL-3 on colony formation by rhesus monkey bone marrow cells (panel A) and the capacity of RhIL-3 to support the production of GM-CSF responsive clonogenic cells in liquid cultures of rhesus monkey bone marrow cells (panel B). The "BM colony formation" assay and the "BM liquid culture" assay are described in section 2.2.4 of chapter 2. Rhesus monkey BM colony formation was stimulated by purified *B. licheniformis* derived RhIL-3. Upon incubation of BM cells in liquid cultures for seven days in the presence of graded concentrations of RhIL-3, the cell cultures were assayed for GM-CSF responsive progenitor cells. Recombinant human GM-CSF was used at supraoptimal concentrations of 5 µg/l.

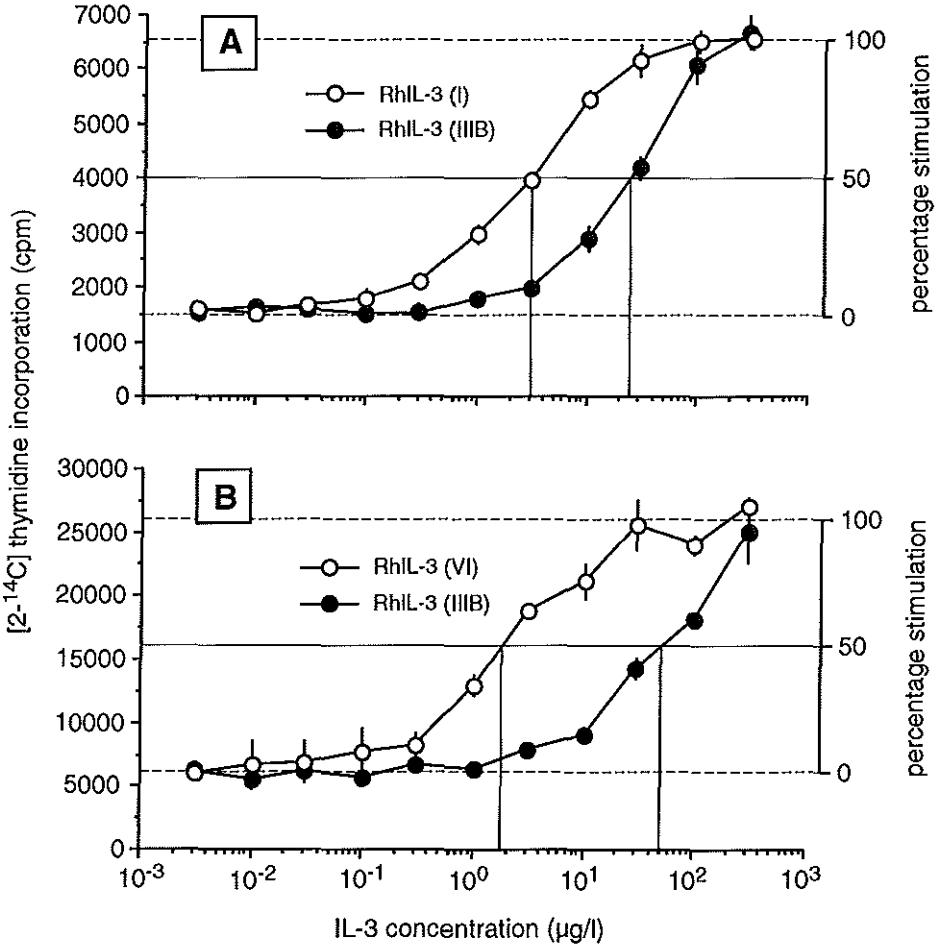


Fig. 5.14 Differential effect of laboratory-scale and intermediate-scale *B. licheniformis* derived RhIL-3 on human AML-193 cells. Panel A shows the differential effect of a representative laboratory-scale produced RhIL-3 preparation (batch I) and the intermediate-scale produced preparation (batch IIIB) and panel B shows the dose-effect relationship of another representative laboratory-scale produced RhIL-3 preparation (batch VI)in comparison to batch IIIB. DNA synthesis in AML-193 cells was measured as detailed in section 2.2.2 of chapter 2.

measured by various biochemical procedures, including reducing and non-reducing SDS-polyacrylamide gel electrophoresis, which indicated similarity of molecular weight and disulfide bridge formation. Western blot analysis indicated no differences in aggregation nor in the abundance of antigenic

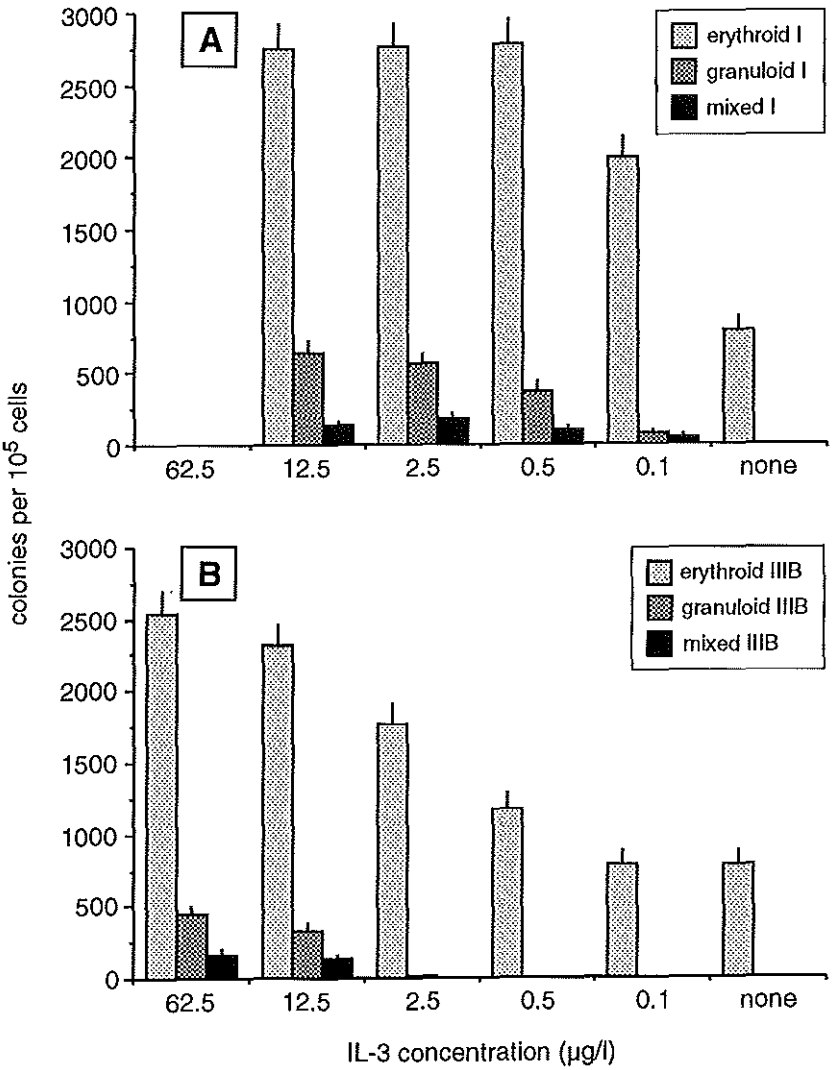


Fig. 5.15 Differential effect of independently produced RhIL-3 preparations (batch I and batch IIIB; panel A and panel B, respectively) on colony formation by purified human bone marrow progenitor cells. Colonies were classified as erythroid, granuloid or mixed (containing at least erythroid plus myeloid cells) according to standard criteria. Numbers of colonies (±SD) were calculated per 10^5 purified human hemopoietic progenitor cells.

determinants. Mass spectrometry indicated an identical molecular weight. Therefore, the *in vitro* biological differences could not be explained by

differences in primary structure and have to be explained by differential tertiary structures. With this respect it is noteworthy that the supernatant of one laboratory-scale fermentation (batch VII) also contained hardly any biological activity, whereas the IL-3 protein concentration, determined by Western blot analysis, did not differ from similarly produced active batches such as batch I, II, V and VI. This indicates that production of inactive RhIL-3 is presumably not dependent on the production process. The inactive RhIL-3 from batch VII failed to bind to the hydrophobic interaction chromatography column (Fractogel TSK butyl 650C, see chapter 2). This finding supports the hypothesis of a rearranged conformation, which apparently precludes binding. In contrast, analysis of nuclear magnetic resonance (NMR) spectra derived from RhIL-3 batch I and batch IIIB revealed that both preparations have virtually identical ^{1}H-NMR spectra (D. Schipper, Royal Gist-Brocades NV, Delft, personal communication). The latter makes it unlikely that differential conformation accounts for the functional difference *in vitro*. It is noteworthy that the spectra indicate that the rhesus monkey IL-3 preparations were homogeneous, i.e, free of interfering molecules, and did not provide indications for C-terminally truncated RhIL-3, due to proteolytic degradation. Furthermore, the two dimensional NOESY spectra of RhIL-3 and hIL-3 were very similar indicating that the overall tertiary structure of hIL-3 and RhIL-3 is the same. The latter could be expected in view of the rather high homology (approximately 80%) found between the protein sequences. Nevertheless, a lack of cross-reactivity was found for these proteins (see next section) contrasting the similarity in primary structure and NMR spectra .

An intriguing observation was made in an attempt to show biochemical protein differences between hIL-3 and RhIL-3 by native electrophoresis. Under standard conditions RhIL-3 migrated to the opposite direction (anode instead of cathode) compared to hIL-3 as demonstrated by reversing the polarity of the electrophoresis system (Fig. 5.16A). Furthermore, RhIL-3 batches subjected to "reversed polarity" native electrophoresis revealed distinct electrophoretic migration patterns (Fig. 5.16B). Batch VI (Fig. 5.16B; lane 3) consisted of an homogeneous slow migrating RhIL-3 protein (indicated by arrow A), whereas Batch IIIB (Fig. 5.16B) consisted predominantly of a much faster migrating RhIL-3 protein (indicated by arrow B). Batch II (Fig. 5.16B; lane 2) consisted of equal concentrations of both forms. Because the molecular weights of both forms are identical, a differential net charge most likely underlies the difference in electrophoretic mobility. As to whether the differential net charge results from modifications such as methylation or acetylation of the protein or alternatively arises from distinct conformations, which apparently could not be unraveled by NMR analysis, remains to be elucidated. Analysis of the biological activity of the distinct proteins, excised and eluted from the gel, revealed that the faster migrating protein (Fig. 5.16B; band B) exerted an 100-fold lower biological activity on the AML-193 cells than the more slowly migrating protein

the diminished activity of RhIL-3 batch IIIB can be explained by the assumption that more than 90% of this preparation is an inactive RhIL-3 polypeptide. It is noteworthy that receptor binding competition studies showed that RhIL-3 batch IIIB, compared with hIL-3, displayed a 100 to 1000-fold decreased capacity to compete for the dual high affinity IL-3/GM-CSF receptor present on human AML monocytes (Budel et al., 1990). RhIL-3 batch I showed a 3- to 10-fold decreased capacity to compete for this receptor (L.M. Budel, Dr Daniel den Hoed Cancer Center, Rotterdam, personal communication).

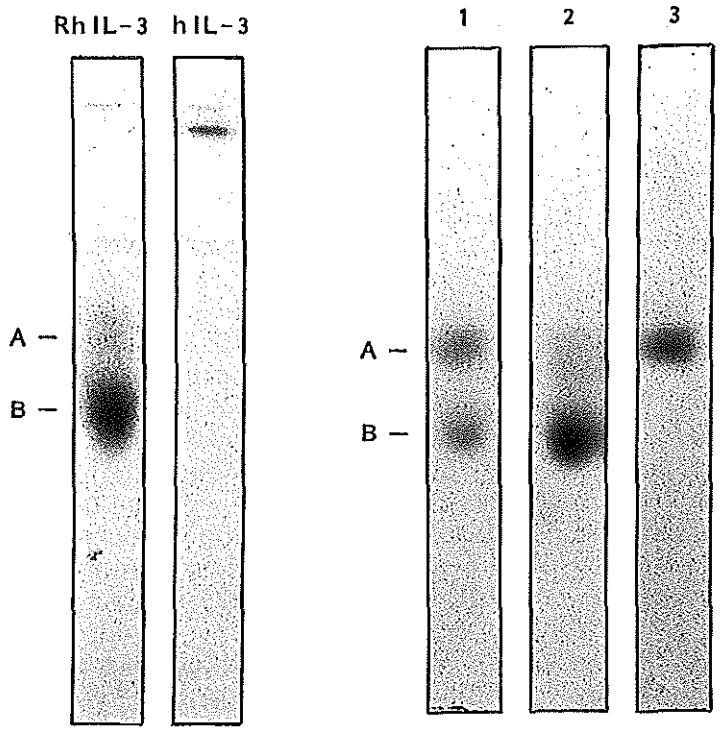


Fig. 5.16 Reversed polarity electrophoresis of purified RhIL-3 preparations. Following native reversed polarity electrophoresis (allowing proteins to migrate to the anode instead of cathode), IL-3 proteins were visualized by Coomassie brilliant blue. Differential electrophoretic mobilities of hIL-3 and RhIL-3 are shown in the left panel. The right panel shows the distinct electrophoretic mobility patterns of batch II (lane 1), batch IIIB (lane 2) and batch VI (lane 3). The more slowly migrating RhIL-3 protein (band A) exerted a 100-fold higher biological activity on AML-193 cells than and the faster migrating RhIL-3 protein (band B).

These observations are in line with the biological data obtained from the AML-193 cells as described here. Therefore, it can be assumed that the *in vitro* biological inactivity of RhIL-3 batch IIIB (predominantly inactive protein corresponding to the faster moving band B) resides in the failure of this RhIL-3 to bind to its receptor.

In contrast to the differences of the biological activities measured *in vitro*, the *in vivo* administration to rhesus monkeys of graded doses RhIL-3 of batch IIIB and batch I resulted in similar dose-effect relationships (Wagemaker and Van Gils, personal communication). The nature of the differences of the *in vitro* and *in vivo* biological properties of RhIL-3 batch IIIB is as yet a matter of speculation. Metabolic conversion *in vivo* of the inactive form into an active configuration might be an reasonable explanation but this hypothesis awaits further investigation.

5.4 Comparative studies between human and rhesus monkey Interleukin-3.

Glycosylated RhIL-3 present in COS[pSVL/RhIL-3] CM displayed biological activity on AML-193 cells (Fig. 5.9). Homogeneous RhIL-3 expressed by *B. licheniformis* exerts its activity on AML-193 cells at concentrations comparable to that of hIL-3 although it is a consistent observation that the specific activity of RhIL-3 is 3-fold less than the specific activity of hIL-3 (Fig. 5.17). Along with the stimulation of human malignant hemopoietic cells, RhIL-3 also promotes colony formation of purified normal human bone marrow cells (Table 5.3). Furthermore, comparable dose-effect relationships were observed using either human or RhIL-3 and differences in colony morphology and or differentiation lineages were not found (Table 5.3). Despite the similar dose-effect relationships displayed in Table 5.3, the results do not exclude the existence of a small difference, such as the 3-fold reduction of stimulatory capacity as observed on AML-193 cells. Therefore, precise dose-effect relationships were established in order to assess the stimulatory activity of human and RhIL-3 on colony formation by purified human bone marrow progenitor cells. Such dose-effect relationships (e.g. Fig. 5.8) displayed that RhIL-3 has a 3-fold lower activity compared to hIL-3 on AML-193 cells (Fig. 5.17). Based on these results it was concluded that RhIL-3 is able to stimulate human hemopoietic cells with a minor reduction in its stimulatory capacity as compared to hIL-3. However, it can not be excluded that the amount of inactive RhIL-3 (Fig. 5.16 band B), present in most batches, accounted for the observed 3-fold decreased stimulatory capacity of RhIL-3 on human hemopoietic cells. However, this is not in concordance with RhIL-3 batch VI (Fig. 5.16 lane 3). While this batch contained homogeneous active RhIL-3, i.e., the slower migrating RhIL-3 protein (indicated as band A in Fig. 5.16), it also showed a 3-fold decreased stimulatory capacity on AML-193 cells.

The different stimulatory activity of human and RhIL-3 on rhesus monkey bone marrow progenitor cells are in contrast with the closely similar effects of human and RhIL-3 on human cells (AML-193 and BM cells). Initially, COS cell derived RhIL-3 and purified RhIL-3 produced by *B. licheniformis* were used for comparative studies between human and RhIL-3. Responses of rhesus monkey bone marrow progenitor cells to identical unit numbers of either hIL-3 or RhIL-3, as determined by using AML-193 cells, suggested a much lower activity of hIL-3 for rhesus monkey cells (Fig. 5.11). The availability of purified unglycosylated B. licheniformis RhIL-3 facilitated comparative studies between homogeneous and identically synthesized human and RhIL-3.

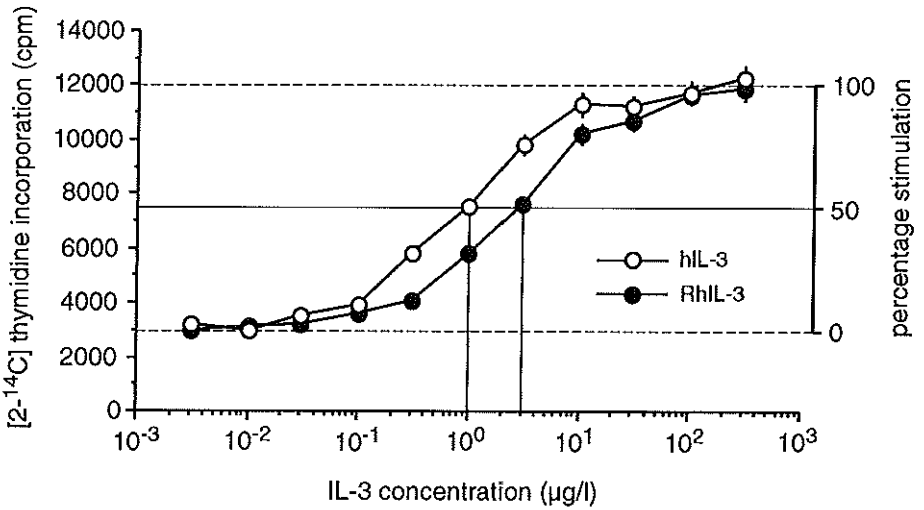


Fig. 5.17 Effect of human IL-3 (hIL-3) and rhesus monkey IL-3 (RhIL-3) on AML-193 cells. Representative dose-effect relationships of hIL-3 and Rh IL-3 are shown and reveal that hIL-3, as compared to RhIL-3, has a 3-fold higher capacity to stimulate AML-193 cells. The concentrations required for half-maximal stimulation of AML cells by hIL-3 and RhIL-3 are indicated (1 and 3 µg/l, respectively).

Stimulation of rhesus monkey bone marrow cells by RhIL-3 appeared to be more effective than by its human homolog (Fig. 5.18). In this particular experiment the CD34 positive rhesus monkey bone marrow progenitor cells were not depleted from MO1 positive cells , and as a consequence "spontaneous" proliferation was not completely eliminated. Apparently, the presence of accessory cells was debet to the limited effect of IL-3. Moreover, it was observed that in those cases of "spontaneous" proliferation (background colony formation) addition of exogenous IL-3 hardly augmented colony formation.

Table 5.3 Effect of human and rhesus monkey IL-3 on colony formation of purified human hemopoietic progenitor cells[a].

IL-3 concentration (ng/ml)	colonies/10^4 (±SD)		
	Erythroid	Granulocytic	Mixed
Rhesus monkey IL-3			
12.5	275 ± 17	64 ± 9	13 ± 4
2.5	276 ± 17	56 ± 8	18 ± 5
0.5	278 ± 18	37 ± 7	10 ± 3
0.1	199 ± 15	8 ± 3	5 ± 2
Human IL-3			
12.5	243 ± 17	60 ± 8	17 ± 5
2.5	239 ± 16	52 ± 8	23 ± 5
0.5	240 ± 16	38 ± 7	6 ± 3
0.1	183 ± 15	21 ± 5	3 ± 2
0	79 ± 10	0	0

[a] Human hemopoietic bone marrow cells were prepared and cultured as detailed in chapter 2. Colonies were classified as erythroid, granulocytic or mixed (containing at least erythroid plus myeloid cells) according to standard criteria (see chapter 2). Colony numbers (±SD) in duplicate cultures of bone marrow cells were calculated per 10^4 purified bone marrow cells.

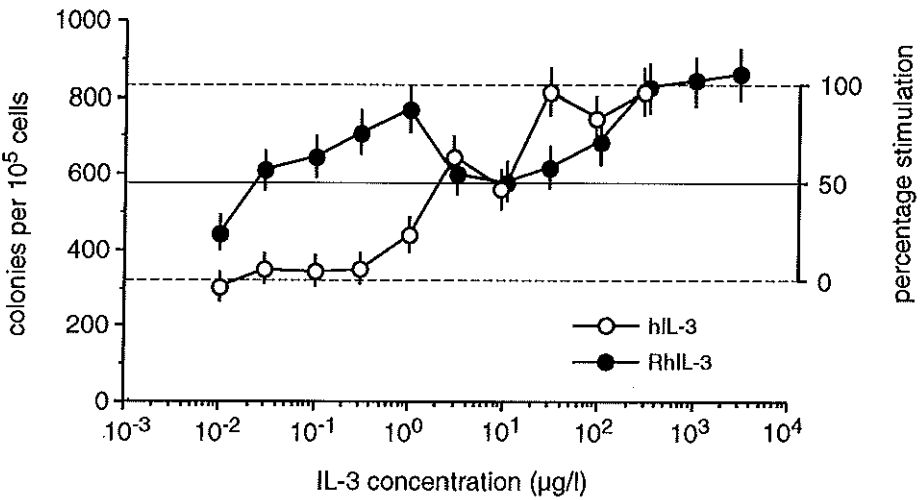


Fig. 5.18 Effect of human and rhesus monkey IL-3 on colony formation by rhesus monkey bone marrow progenitor cells. Purified rhesus monkey BM progenitors were not depleted from accessory cells in this particular experiment, which explains the high background colony formation (dotted line). Numbers of colonies (±SD) were calculated per 10^5 purified rhesus monkey hemopoietic progenitor cells.

Furthermore, it has been recognized that the response to IL-3 of purified populations of human hemopoietic bone marrow progenitor cells in the complete absence of accessory cells is significantly impaired (Bot et al., 1988). Therefore, selected doses of MO1 positive cells, prepared as described in chapter 2, were added to cultures containing highly purified rhesus monkey bone marrow progenitor cells. RhIL-3 appeared to be at least 100-fold more effective than its human homolog in the stimulation of rhesus monkey bone marrow derived, T lymphocyte depleted, MO1 negative and either CD34 positive or HLA-DR positive stem cell concentrates (for purification procedures: see section 2.2.5 of chapter 2).

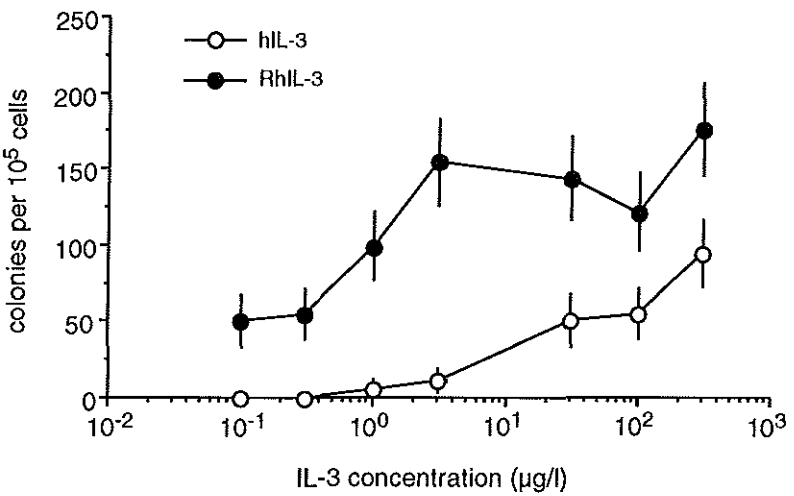


Fig. 5.19 Effect of human and rhesus monkey IL-3 on colony formation by highly purified and MO1 depleted rhesus monkey bone marrow cells. Numbers of colonies (±SD) were calculated per 10^5 purified rhesus monkey hemopoietic progenitor cells.

An example of such an experiment is shown in Fig. 5.19. These highly purified BM progenitor cells were supplemented with appropriate numbers of MO1 positive cells to allow colony formation upon addition of exogenous HGFs. Spontaneous proliferation of progenitors without addition of IL-3 was not observed in these rhesus monkey bone marrow cultures indicating that the presence of a small number of accessory cells, capable of producing endogenous hemopoietic growth factors, did not interfere with IL-3 dependent colony formation. Thus, the results obtained from comparative studies between human and RhIL-3, presented in this section, demonstrate a species specificity of IL-3. It should be emphasized that the lower capacity of hIL-3 as compared to RhIL-3 (± 100 fold) to stimulate rhesus monkey bone marrow cells is much more profound than the marginal difference of stimulatory capacity observed between human and RhIL-3 (± 3 fold) on human cells, indicating a preferential unidirectional species specificity. Recently, it was shown that hIL-3 compared to RhIL-3 has a 20- to 30-fold lower affinity for the IL-3 receptor present on normal rhesus monkey BM cells(Van Gils et al., 1994a). In contrast, only a small difference in affinity was found between hIL-3 and RhIL-3 with respect to relative binding to IL-3 cell surface receptors on human AML blast cells. Thus, the binding characteristics of human and RhIL-3 on human and rhesus monkey cells supported the presence of an functional species specificity. The decreased affinity of hIL-3 for the rhesus monkey IL-3 receptor (Van Gils et al., 1994a) explains the limited effects of hIL-3 when tested in *Macaca* species. The results of these IL-3 binding studies confirmed that the observed species-specificity is largely unidirectional.

The *in vivo* effects of RhIL-3 were assessed by administration of RhIL-3 to normal rhesus monkeys. Administration of homologous IL-3 resulted in a rapid increase of bone marrow progenitor cells, followed by a rise of circulating progenitor cells, and a vast increase of all bone marrow derived blood cell types (Wagemaker et al., 1990b). Direct *in vivo* comparison of human and RhIL-3, administered to rhesus monkeys confirmed the relatively weak capacity of hIL-3 to elicit a hemopoietic response in nonhuman primates belonging to the *Macaca* species (Van Gils et al., 1994a). Fig. 5.20 shows the effect on the numbers of nucleated cells in response to hIL-3 and RhIL-3 administration (30 µg/kg/day intravenous) for 17 consecutive days. After a lag phase of one week following homologous IL-3 (RhIL-3) administration, increases in numbers of eosinophilic and neutrophilic granulocytes and the appearance of large numbers of cells designated as atypical (basophilic) granulocytes, reported earlier (Donahue et al., 1988; Mayer et al., 1989) were observed (Fig. 5.20 panel B). In contrast to RhIL-3, analogous administration of hIL-3 to rhesus monkeys showed hardly an effect on the numbers of nucleated blood cells (Fig. 5.20 panel A). These *in vivo* data convincingly demonstrate that the effects of hIL-3 in nonhuman primates are considerably impaired by the functional species specificity as described in this section.

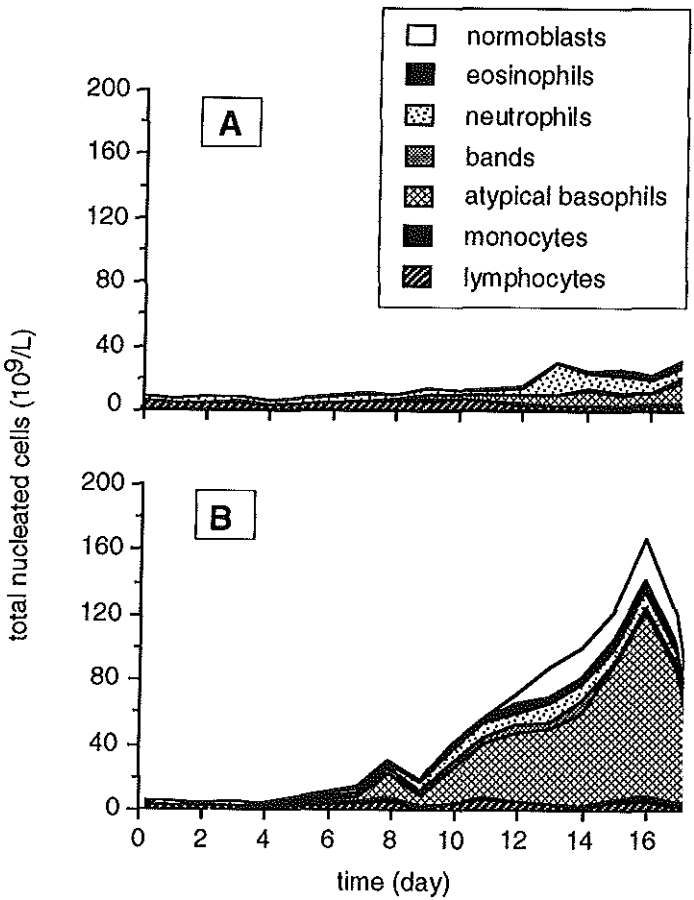


Fig. 5.20 Differential effect of human (panel A) and rhesus monkey IL-3 (panel B) on peripheral nucleated blood cell counts in rhesus monkeys. IL-3 was administered to normal rhesus monkeys by continuous iv infusion of 30 μg/kg/day during 17 consecutive days (Van Gils et al., 1994a). Normoblasts, eosinophils, neutrophils, bands, atypical basophils, monocytes, and lymphocytes are shown in both panels from top to bottom. The monkey that had received homologous IL-3 was lethally irradiated at day 17 to investigate the effect of IL-3 priming on bone marrow recovery after transplantation. This implicated that the comparative study between human and rhesus monkey IL-3 was terminated at day 17.

5.5 Summary and Discussion

In this chapter the *in vitro* effects of a novel human factor encoded by the

cDNA clone D11 on hemopoietic cells were investigated. Following the cloning and expression of cDNA clone D11 in COS cells (see chapter 3 and Dorssers et al., 1987), the biological activity demonstrated that the isolated factor stimulates human bone marrow progenitors committed to several hemopoietic differentiation lineages (Dorssers et al., 1987; Bot et al., 1988). Thus, the novel factor is functionally as well as genetically and biochemically related to rodent IL-3 and therefore it is has been designated human interleukin-3 (hIL-3).

The spectrum of biological activities of murine IL-3 has been extensively studied (reviewed by Ihle et al., 1989). These studies showed that murine IL-3 supports the proliferation of multipotent and committed progenitor cells. The availability of homogeneous hIL-3 (see chapter 3) allowed studies on human hemopoietic cell populations, including normal human bone marrow cells as well as malignant hemopoietic cells or cell lines. Preliminary characterization of hIL-3 using COS[pLB4] CM revealed that this factor exerts biological effects on human BM cells. In line with these COS[pLB4] CM studies, recombinant homogeneous hIL-3, irrespective of the type of expression system, stimulates the proliferation of human hemopoietic bone marrow progenitor cells and supports the production of bone marrow progenitor cells committed to all myeloid-derived lineages. Whether hIL-3 acts on the earliest pluripotent stem cell and supports renewal of all progenitor cells awaits further study. However, replating experiments using blast cell colonies derived from a progenitor cell enriched population by fluorescence-activated cell sorting with the monoclonal antibody 3C5, showed that IL-3 is able to support the proliferation of a progenitor cell ancestral to the cell from which mixed colonies are derived (Barber et al., 1989). The data presented by these investigators support the concept that IL-3 acts on an earlier hemopoietic cell population than GM-CSF. This concept was further supported by the observation that IL-3 and GM-CSF have a differential stimulatory capacity on the formation of multilineage colonies and blast cell colonies from human bone marrow cells (Leary et al., 1987).

From comparative studies between unfractionated bone marrow and purified progenitor cell populations, it became apparent that the outgrowth of IL-3 stimulated bone marrow progenitors depends on the presence of more linage-specific growth factors such as GM-CSF or erythropoietin. This phenomenon can be mimicked by the addition of minimal numbers of monocytic accessory cells (Bot et al., 1988). Further studies are required to distinguish between responsible mechanisms such as the production of growth factors by these monocytes (in)dependent of hIL-3 or cell-cell interactions between monocytes and colony-forming progenitor cells.

Along with the stimulation of normal hemopoietic cells, hIL-3 strongly stimulates the *in vitro* proliferation of human leukemic blast cells as presented in this chapter. Therefore, the availability of recombinant hIL-3 facilitated assessment of growth factor responses of AML precursors. This CSF

phenotyping (Delwel et al., 1987/1988) may be a useful addition to the morphological classification of AML and the diverse CSF response patterns may reflect the response of normal hemopoietic progenitors at different maturation stages to these stimuli. However, a correlation between FAB classification and the CSF response patterns has as yet not been found. In addition to human AML blasts, clonogenic progenitors derived from other human bone marrow-associated disorders such as myelodysplastic syndromes and myeloproliferative disorders are responsive to hIL-3 (Barber et al., 1989) and growth and differentiation of B cell-associated multiple myelocytic (MM) precursors are under the synergistic control of hIL-3 and IL-6 (Bergui et al., 1989).

As demonstrated in this chapter a wide range of host organisms can be used for the heterologous expression and production of functionally active hIL-3, including bacteria, yeast and mammalian cells. Nonetheless, host organisms differ in productivity, reproducibility, post translational modification, secretion, etcetera. To enable large-scale production of biologically active hIL-3, required to study its therapeutic applications, several expression systems have been examined and their (dis)advantages will be discussed in the next paragraphs.

Expression of hIL-3 in *E. coli* as a fusion protein enables the production of milligram quantities of hIL-3 fusion protein per liter of bacterial culture (Table 5.2). The hIL-3 fusion proteins appeared to be functional in the various bioassays demonstrating that glycosylation is not necessary for biological activity, at least *in vitro*. Furthermore, the hIL-3 fusion protein encoded by pUC/hMultiΔ2 appeared to be biologically active demonstrating that the first 14 N-terminal residues are not required for biological activity. Alternatively, the function of the N-terminal residues have been replaced by the lacZ derived amino acids. Notably, truncated mature hIL-3 lacking 14 N-terminal and 15 C-terminal residues exerted full biological activity. The latter demonstrated that elimination of 15 C-terminal residues, in addition to the 14 N-terminal amino acids, did not affect the stimulatory capacity of hIL-3 (Dorssers et al., 1991). Although not deleterious for *in vitro* biological activity, the heterologous N-terminal residues of the hIL-3 fusion proteins (Table 5.1 and 5.2) made these hIL-3 proteins not particularly suitable as therapeutic agents. Comparable constructs that circumvent the presence of lacZ residues by deletion of their DNA sequences show comparable biological activity but concomitantly a substantially decreased expression.

K. lactis derived hIL-3 showed biological activity at about 5 x 10^{-11} M. This specific activity of yeast derived glycosylated hIL-3 is comparable to that observed for *E. coli* derived hIL-3. The aberrant glycosylation of this yeast derived hIL-3 protein does not influence its *in vitro* biological activity but may affect its function, antigenicity and stability *in vivo*. Furthermore, the yeast synthesized product lacks the first two amino acids which also does not interfere with its biological activity.

Analysis of the biological activity of hIL-3 synthesized in *B. licheniformis*

showed its efficacy on hemopoietic cells to be comparable to the yeast and E. coli derived products. The simple and efficient purification protocol resulted in preparations of homogeneous mature hIL-3. This formulated product is free of contaminating bacterial proteins and DNA. In addition, endotoxin concentrations of these hIL-3 preparations were below the detection level of the assay (Limulus Lysate Assay). Based on these results, it was decided that the *B. licheniformis* derived hIL-3 is the prime candidate to examine the therapeutic applications of hIL-3 (Van Leen et al., 1990; Van Leen et al., 1991). Currently, a Phase I clinical trial with this formulated product is conducted in The Netherlands. It comprises a safety and dose-finding study in which IL-3 is administered following chemotherapy in patients suffering from small cell lung carcinoma. The potential clinical applications of recombinant hIL-3 and the results of the first clinical trials in human patients (Ganser et al., 1990a,b,c) will be discussed in chapter 7.

Mammalian cells such as COS, CHO and C127 cells are able to synthesize hIL-3 in its most natural form, carrying the complex type of glycosylation characteristic for higher eukaryotes. COS cells synthesize 10^4 to 10^5 units per liter, whereas C127 cells can produce up to 10^7 units per liter. As to whether the specific activity of the eukaryotic expressed hIL-3 differs from that found for yeast and bacterial derived IL-3 awaits further investigation. However, assessment of the biological activity of partially purified glycosylated hIL-3 derived from C127 cells on AML-193 suggests a specific activity comparable to those established for yeast and bacterial derived hIL-3. The content of recombinant IL-3 in the partially purified hIL-3 preparations was estimated by SDS-polyacrylamide gel electrophoresis and Western blot analysis and subsequently used to calculate the specific activity which appeared to be 10^6 units per mg IL-3 protein.

Therefore, homogeneous hIL-3, irrespective of content or type of glycosylation, stimulates hemopoietic cell proliferation *in vitro* at approximately 5 x 10^{-11} M concentration. The other human CSFs, including M-CSF, GM-CSF, G-CSF and Epo exert their biological actions *in vitro* at concentrations between 10^{-11} M to 10^{-13} M (Coulombel, 1989). Thus, the specific activity of recombinant glycosylated and unglycosylated hIL-3 is comparable to those found for the other human CSFs. As to whether this specific activity resembles the specific activity of the native hIL-3 remains to be elucidated. This question awaits further intensive study on native hIL-3 and its purification to homogeneity which is hindered by the lack of abundant natural hIL-3 sources.

Data presented in this chapter demonstrate that rhesus monkey IL-3 (RhIL-3) is effective on human as well as rhesus monkey hemopoietic cells, at least *in vitro*. The availability of functional RhIL-3 facilitate preclinical *in vivo* studies on homologous IL-3 in nonhuman primates. Administration of homologous IL-3 to nonhuman primates avoids a possible functional species barrier and may diminish immunological responses. However, prolonged continuous intravenous

administration of high dose recombinant nonglycosylated homologous IL-3 to rhesus monkeys resulted in a decreased response to IL-3 and a simultaneous rise in titer of IL-3 specific antibodies (Van Gils et al., 1994b). This study suggested that nonglycosylated IL-3 may elicit a neutralizing antibody response.

In spite of approximately 80% homology of the amino acid sequences of hIL-3 and RhIL-3, comparison of their biological activities revealed differential stimulatory activity, especially on rhesus monkey bone marrow cells. RhIL-3 appeared to be about 100-fold more effective in stimulating rhesus monkey hemopoietic progenitors than hIL-3, whereas comparable stimulatory activity was observed on normal as well as malignant human hemopoietic cells. The much lower capacity of hIL-3 to support colony formation by rhesus monkey progenitor cells demonstrates a species specificity of hIL-3. Assessment of biological activities of recombinant hIL-3 synthesized by various microorganisms all yielded similar dose-effect relationships, as presented in this chapter. Apparently, the type and extent of glycosylation does not alter the specific activity of hIL-3 which is in agreement with reports on mouse IL-3 (Kindler et al., 1986; Metcalf et al., 1986). In addition, it was demonstrated in this chapter that glycosylated COS cell derived RhIL-3 and nonglycosylated RhIL-3 synthesized in *B. licheniformis* were equally effective on human AML-193 cells and rhesus monkey bone marrow cells. Furthermore, it should be emphasized that the specific activity of B. licheniformis derived RhIL-3 assessed on rhesus monkey bone marrow was identical to that found for hIL-3 on human bone marrow. Based on these data we have no reason to assume that the diminished capacity of hIL-3 to support colony formation by rhesus monkey bone marrow progenitor cells may be attributed to differential post translational processing, since there is no evidence that differential processing influences the biological activity of mouse, rhesus monkey and human IL-3. Since endotoxin was not detected in the *B. licheniformis* derived homogeneous IL-3 preparations, the observed species specificity could not be the result of indirect IL-3 effects such as endotoxin mediated growth factor production by bone marrow cells.

The nature of the species specificity of hIL-3 is as yet a matter of speculation. However, the unusual high number of substitutions at amino acid replacement sites, as presented in the previous chapter, indicates that IL-3 has been subject to a high evolution rate. With regard to the cross-reactivity of IL-3, it is noteworthy that human and mouse IL-3 do not show any cross reactivity (Dorssers et al., 1987) and neither do mouse and rat IL-3 (Cohen et al., 1986). In addition, human IL-3 fails to stimulate rat bone marrow cells (unpublished observation). A remarkable observation was made by Barber et al. (1989), these investigators demonstrate that human GM-CSF effectively stimulated sheep bone marrow cells (a cross-reactivity not observed between man and mice), whereas sheep bone marrow did not respond to hIL-3 in both proliferation and clonal assays. Apparently, IL-3 exhibits a more pronounced species specificity

than most other hemopoietic growth factors which is in line with the low IL-3 protein homology observed among species. With respect to human and murine IL-3, the protein homology (28%) is much lower than that observed for other HGFs (see chapter 3, section 3.12) which varied from 42% (IL-6) to 90% (IL-11). Pairwise comparison of IL-3 coding sequences of various primate and rodent species will be discussed in the next chapter.

The species specificity of hIL-3 as encountered for rhesus monkey cells also implies that preclinical *in vivo* studies in cynomolgus and rhesus monkeys (Donahue et al., 1988; Krumwieh and Seiler, 1989; Mayer et al., 1989; Umemura et al., 1989) might very well be insufficiently representative for the effects of hIL-3 in human patients. In fact, the effects of hIL-3 observed in these *Macaca* species were restricted to a small rise in white blood cells, predominantly caused by basophilia, whereas the first clinical trial in human patients showed rises in neutrophilic and eosinophilic granulocytes as well as elevated thrombocyte numbers. *In vivo* administration of homologous IL-3 to rhesus monkeys resulted in a dose-dependent stimulation of the production of all bone marrow derived cell lineages (Wagemaker et al., 1990b). Comparative *in vivo* analysis between hIL-3 and RhIL-3 in rhesus monkeys (Van Gils et al 1994a), together with the *in vitro* results as described and presented in this chapter, convincingly demonstrate that stimulation of rhesus monkeys by hIL-3 is considerably impaired by a species specificity. Neither absolute cell numbers nor the variety of cell types produced have been observed in studies with hIL-3 in *Macaca* species indicating that those studies were substantially influenced by the decreased activity of hIL-3 on rhesus monkey cells. Thus, the observed results from preclinical studies using hIL-3 in *Macaca* species are not representative for the full potential of IL-3.

CHAPTER 6

MOLECULAR EVOLUTION OF INTERLEUKIN-3

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*Burger, H., M.C. Mostert, E.M. Kok, G. Wagemaker and L.C.J. Dorssers. Cloning and expression of interleukin-3 genes of chimpanzee and new world monkeys. BBA **1217**: 195-198, 1994.*

Burger, H., G. Wagemaker, J.A.M. Leunissen and L.C.J. Dorssers. Molecular evolution and species specificity of interleukin-3. J. Mol. Evol. 39: 255-267, 1994.

Dorssers, L.C.J., H. Burger, G. Wagemaker and J.P. de Koning. Identification of functional domains of interleukin-3 by construction of primate interspecies chimera. Growth Factors 11: 93-104, 1994.

6.1 Introduction

The rapid accumulation of nucleotide (nt) and amino acid (aa) sequence data in the last two decades has stimulated studies to explore and establish evolutionary relationships. Such comparative analyses on a molecular basis, usually referred to as "molecular evolution" provide new insights in the nature of the evolutionary process. Until recently, evolutionary biology was mainly explored by biologists working at the level of whole organisms. Since the invention of molecular evolution, evolutionary biology is not a discipline of concern only to anthropologists, geologists, paleontologists, ecologists and zoologists but also to investigators working in the field of cell biology, molecular biology, developmental biology, physiology and behavioral biology. Thus, evolutionary biology has evolved into a broad multi-disciplinary pursuit that through molecular evolution probes evolution at its most basic molecular level by direct exploration of changes occurring in genes. The study of evolutionary relationships between homologous protein or nucleic acid sequences was rapidly integrated into the disciplines of taxonomy, paleontology and geology. Until that time, such relationships were primarily based on fossil evidence.

Comparative analyses of DNA and protein sequences are increasingly important and helpful components in evolutionary biology. Initially, systematic sequence comparisons were restricted to protein sequence data. Based on similarities between contemporary protein sequences, it was postulated that all proteins are descendants from a small number of prototypes (Doolittle, 1981). This premise was based on the notion that it is more simple to duplicate and modify proteins genetically than it is to assemble proteins *de novo* from random beginnings. Therefore, the ultimate goal of early protein evolutionists was the reconstruction of past events that have resulted in present-day protein sequences. After the development of methods for routine nucleotide sequence determination (Maniatis et al., 1975), comparative analyses of coding and non-coding DNA sequences were included in the study of molecular evolution. Critical elements of molecular evolution are point mutations, i.e., single replacements of nucleotides (bases) in DNA, and regulatory mutations, i.e., changes in a gene or in the vicinity of a gene which influence its expression. It has been proposed that regulatory mutations are critical elements in the morphological evolution of organisms (Wilson, 1985; Zuckerkandl, 1987). Thus, morphological changes are likely to be attributable to changes in gene regulation, particularly those that turn on or off specific genes in the course of embryonic development. A better understanding of the molecular basis of embryonic development is required to identify the genes whose differences account for the anatomical differences between species of multicellular organisms. In contrast to regulatory mutations influencing the expression level of a gene, point mutations may affect the structure of a particular gene product. However, the link between molecular and organismal evolution is likely to be

established by the examination of regulatory mutations rather than that of point mutations.

Point mutations in the coding region of a gene can affect the aa sequence of the encoded protein and thereby its function. Analyses of point mutations has increased understanding of evolutionary processes and yielded many important insights into the branching of species. Initial examination of point mutations has resulted in the conceptualization of the molecular clock, i.e., the establishment of a clock-like linear relation between the number of mutations and the elapsed time of divergence. (Zuckerkandl and Pauling, 1965). Moreover, the molecular clock hypothesis postulates that the mutation rate is equal for all organisms and is approximately constant over time.

Initially, it was suggested that nucleotide substitutions occur randomly and with the same rate at every position of the DNA molecule (Kimura, 1968). The rapid accumulation of DNA sequence data demonstrated nonrandom patterns of nt substitutions. The rate of evolution at parts of the DNA molecule that directly affect the function of a protein is generally much slower as compared to sites that do not affect its function. This concept of functional constraint, i.e., a slow evolutionary change appears to be associated with strong functional constraint and vice versa, has also been demonstrated for the different positions in a codon, i.e., a nt triplet specifying a particular aa (Kimura, 1977, Miyata et al 1980). The rate of evolutionary change at the third position of a codon is higher than the rate at the second position of a codon. This observation corresponds to the fact that whereas any base change at the second position results in an aa substitution, about half of the base changes at the third position do not result in an aa substitution. Therefore, the coincidence that nt substitutions result in aa replacements which may affect the function of a protein is reciprocal to the observed rates at the different positions of a codon, thus in line with the concept of functional constraint.

Several methods have been devised in order to estimate the number of substitutions causing an aa replacement and the number of silent substitutions (no aa change) separately (Kimura, 1980; Miyata and Yasunaga, 1980; Perler et al., 1980; Li et al., 1985). Such an approach is desirable when the amino acid coding region of genes are compared. In the study presented here, we used the method of Li et al. (1985) to estimate the number of synonymous substitutions (nt changes which do not result in an aa replacement) and nonsynonymous substitutions (nt changes resulting in an aa change) between the coding regions of homologous IL-3 genes. The approach of Li et al. (1985) includes corrections for multiple hits at one site (superimposed substitutions) and the algorithm takes into account the differences in substitution rates between transversions and transitions and the relative likelihood of codon interchanges (see also section 2.4 of chapter 2).

The IL-3 nucleotide (nt) and amino acid (aa) sequences of mouse (Fung et al., 1984; Yokota et al., 1984; Miyatake et al., 1985; Campbell et al., 1985;

Kindler et al., 1986), rat (Cohen et al., 1986), gibbon (Yang et al., 1986), rhesus monkey (see chapter 4; Burger et al., 1990a,b), and man (see chapter 3; Yang et al., 1986; Dorssers et al., 1987; Otsuka et al., 1988) have been identified by molecular cloning. The established DNA and protein IL-3 sequences of these various species enabled comparative analyses of their primary structure (comparison of their nucleotide and amino acid sequences) and comparison of their predicted secondary structures (α helix, β-plated sheet, turn and random coil distribution). In addition, tertiary or 3-dimensional structure related parameters such as the hydrophobicity and hydrophylicity distribution, termed the hydropathy profile, of the various IL-3 species can be analyzed. A prerequisite to perform such comparative analyses is a multiple sequence alignment of the IL-3 sequences. In addition, such a multiple sequence alignment allows the construction of phylogenetic trees. Phylogenies from homologous protein or nucleic acids sequences reflect the evolutionary relationships between the species from which the sequences were derived. With respect to the homologous IL-3 sequences, such a phylogenetic tree will be used to calculate the rate of evolution in all branches, rather than to reconstruct the already widely adopted tree topology and times of divergence among the lineages of human (*Homo sapiens*), gibbon (*Hylobates*) and rhesus monkey (*Macaca mulatta*), all genealogically classified in the infraorder of the Catarrhini (Pilbeam, 1984; Andrews, 1985) and the mouse (*Mus*) and rat (*Rattus*) both classified in the infraorder of the Muridae which belong to the Rodentia, a suborder of the Myomorpha (Li and Tanimura, 1987).

Preliminary data obtained from comparative studies between natural IL-3 variants from various species indicated differential substitution rates and highly variable distribution of synonymous and nonsynonymous substitutions among the various lineages (as described in the next sections of this chapter). However, the number of operational taxonomic units (OTUs), i.e., the species for which actual data were available, was not sufficient to study the peculiar molecular evolution of IL-3 in detail. Furthermore, the IL-3 sequences of the rodents and primates were diverged to such an extent that the construction of a reliable alignment and phylogeny was not feasible. Therefore, we decided to identify the IL-3 nucleotide sequences of the *Saguinus oedipus* (crested tamarin) and the *Callithrix jacchus* (common marmoset), both classified in the Callitrichidae, a family of the infraorder of the Platyrrhini ("New World monkeys"). These Platyrrhini sequences would be much closer outgroups to the Catarrhini (Old World monkey, ape, and human) sequences than are the rodent sequences and may further be useful to construct the exact multiple sequence alignment of all the established IL-3 coding sequences. In addition, the chimpanzee (*Pan troglodytes*) IL-3 gene was identified because it facilitated the analyses of recent branches from hominoid evolution.

6.2 Identification and characterization of the nucleotide sequences of the chimpanzee, tamarin and marmoset IL-3 coding regions

Chimpanzee and marmoset chromosomal DNA were extracted from mononucleated peripheral blood cells derived from monkeys held at the Primate Center in Rijswijk, The Netherlands. Tamarin genomic DNA was isolated from the Epstein-Barr Virus (EBV) transformed B-cell line B95-8 (Miller et al., 1972; ATCC CRL1612). Molecular cloning of the IL-3 genes of these three species was accomplished by DNA amplification (PCR: polymerase chain reaction) using amplimers located in the promoter region and just downstream of the translation termination codon (see also section 2.1.4.5 of chapter 2). The PCR strategy and the IL-3 specific oligonucleotide primers used to identify the tamarin, marmoset and chimpanzee IL-3 coding regions are shown in Fig. 6.1.

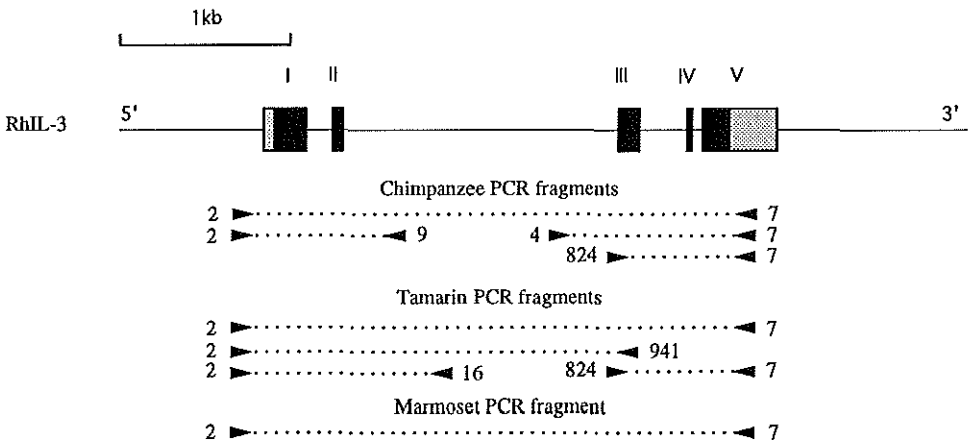
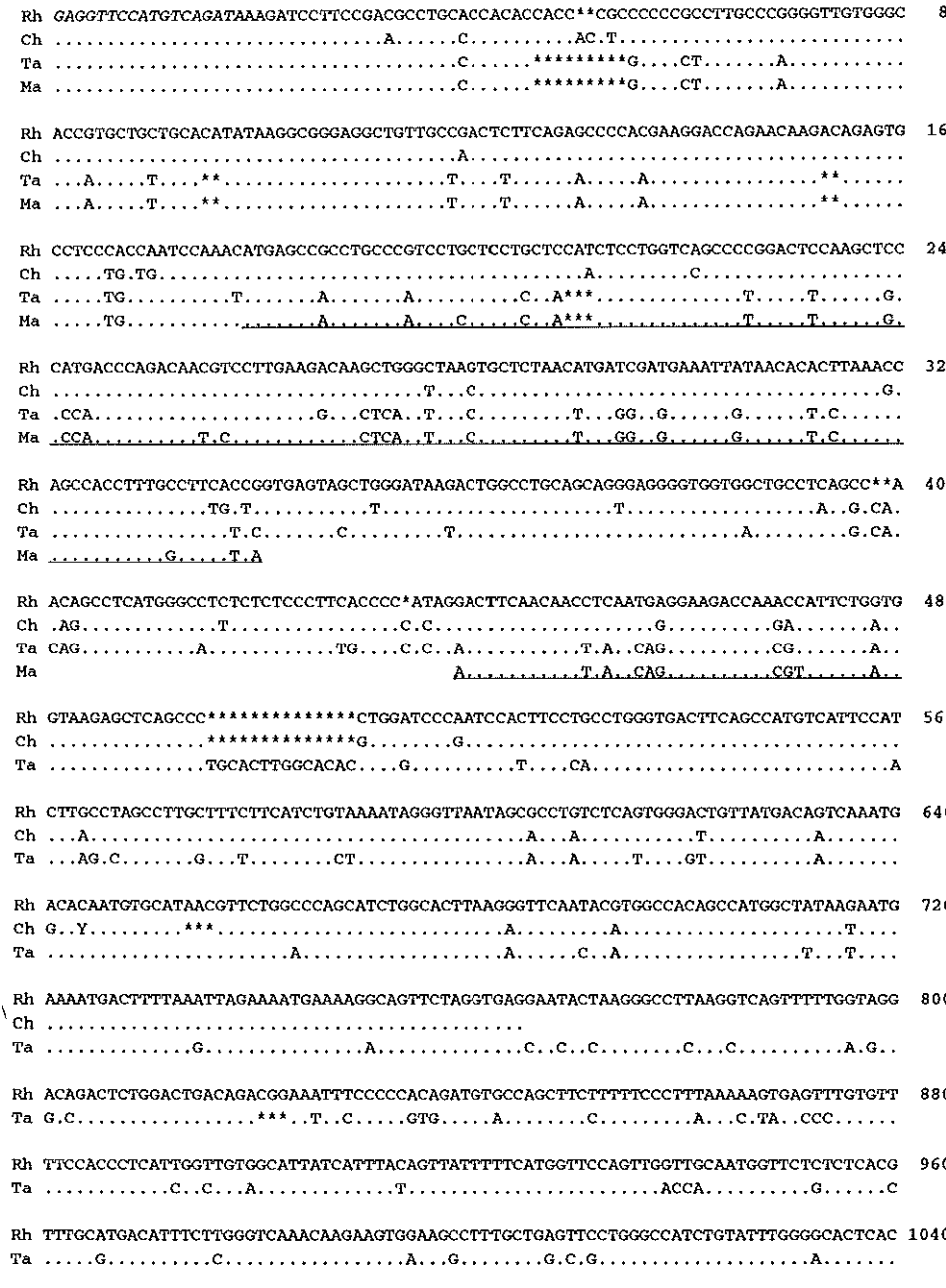


Fig. 6.1 Schematic representation of the PCR strategy to identify the chimpanzee, tamarin and marmoset IL-3 genes. The genomic organization of the rhesus monkey IL-3 (RhIL-3) gene is shown at the top. Black boxes indicate coding regions of exon I-V, whereas dotted areas represent the noncoding regions of exon I and V). IL-3 specific amplimers (see chapter 2) were used to amplify the corresponding chimpanzee, tamarin and marmoset IL-3 sequences. The location of the various primers are indicated. Primer 2 (corresponding to nt 1109-1128 of the RhIL-3 gene; see Fig. 4.4) and primer 7 (corresponding to nt 3362-3380; Fig. 4.4), located in the promoter region and just downstream of the translation termination codon, respectively. The various amplified fragments are indicated.

Amplified fragments of approximately 2.3 and 2.1 kb (from at least two independent PCR reactions) were isolated from the chimpanzee and the two species of the New World monkeys, respectively. With respect to the chimpanzee IL-3 gene, the nt sequence of two fragments (750 bp and 820 bp), corresponding to nt 1109-1857 of the RhIL-3 gene (this fragment encompasses exons 1 and 2; see for nt positions Fig. 4.4) and nt 2544 through 3363 (this fragment encompasses exons 3, 4, and the complete coding region of exon 5) respectively, have been determined. In case of the tamarin, the nucleotide sequences corresponding to positions 1109-2174 and 2435-3363 of the RhIL-3 gene have been established. The length of the latter fragment appeared to be about 170 bp shorter than the corresponding regions of other primate IL-3 genes such as those identified in man, chimpanzee, and rhesus monkey. Sequence analysis revealed that intron 2 lacks 230 bp upstream of exon 3. Instead of the missing bp, intron 2 of the tamarin IL-3 gene contains 60 bp which do not display significant homology to any of the established IL-3 species. The relevant amplicons (PCR products using primers 2 and 7; see Fig. 6.1) in tamarin and marmoset (the marmoset genomic fragment has not been sequenced) were identical in size, indicating that the marmoset gene most likely contains the same deletion in intron 2 in the vicinity of exon 3. The deletion occurred during the evolution of the Callitrichidae, or, alternatively, the Old World Monkeys (Catarrhini) gained DNA during primate evolution through insertional mutagenesis after their split from the New World monkeys (Platyrrhini). Thus, the exchange or trans-position of DNA (230 bp missing and 60 unidentified bp inserted at the same location) indicate recombination events during early primate IL-3 evolution. The partially identified nt sequences of the chimpanzee, tamarin and marmoset IL-3 genes were aligned to the corresponding RhIL-3 sequences as shown in Fig. 6.2. These primate IL-3 genes showed strong conservation of the intron/exon structure (deduced from the RhIL-3 gene) and revealed extensive sequence homology. DNA sequence similarities of the newly identified primate IL-3 genes with the corresponding regions of the RhIL-3 gene are 94.1% (Ch/Rh; 1570 nt), 84.6% (Ta/Rh; 1803 nt), and 84.8% (Ma/Rh; 623 nt), respectively. The sequence similarities among the newly identified primate IL-3 genes are 86.4% (Ch/Ta; 1560 nt), 83% (Ch/Ma; 625 nt), and 96.6% (Ta/Ma; 621 nt), respectively.

Restriction enzyme analysis and nucleotide sequence analysis of chimpanzee and tamarin genomic fragments suggested that these species exhibited a genomic organization identical to that of other identified IL-3 species. To confirm the deduced intron/exon structures and to asses whether these fragments encode functional proteins, chimpanzee, marmoset and tamarin amplicons (primers 2 and 7) were introduced downstream of a cytomegalovirus promoter in the pCMV-4 vector (Andersson et al., 1989) which contains a SV40 origin of replication allowing transient expression in COS cells.


Rh GAGGTTCCATGTCAGATAAAGATCCTTCCGACGCCTGCACCACACCACC**CGCCCCCCGCCTTGCCCGGGGTTGTGGGC 80
Ch .....A.....C.....AC.T.....
Ta .....C.....*********G....CT.....A.....
Ma .....C.....*********G....CT.....A.....
Rh ACCGTGCTGCTGCACATATAAGGCGGGAGGCTGTTGCCGACTCTTCAGAGCCCCACGAAGGACCAGAACAAGACAGAGTG 160
Ch .....A.....
Ta ...A.....T....**.....T....T.....A.....A.....**.....
Ma ...A.....T....**.....T....T.....A.....A.....**.....
Rh CCTCCCACCAATCCAAACATGAGCCGCCTGCCCGTCCTGCTCCTGCTCCATCTCCTGGTCAGCCCCGGACTCCAAGCTCC 240
Ch .....TG.TG.....A.....C.....
Ta .....TG.....T.....A.....A.....C..A***.....T.....T.....G.
Ma .....TG.....A.....A.....C.....C..A***.....T.....T.....G.
Rh CATGACCCAGACAACGTCCTTGAAGACAAGCTGGGCTAAGTGCTCTAACATGATCGATGAAATTATAACACACTTAAACC 320
Ch .....T...C.....G.
Ta .CCA.....G...CTCA..T...C.....T...GG..G.....G.....T.C.....
Ma .CCA.....T.C.....CTCA..T...C.....T...GG..G.....G.....T.C.....
Rh AGCCACCTTTGCCTTCACCGGTGAGTAGCTGGGATAAGACTGGCCTGCAGCAGGGAGGGGTGGTGGCTGCCTCAGCC**A 400
Ch .....TG.T.....T.....T.....A..G.CA.
Ta .....T.C.....C.....T.....A.....G.CA.
Ma .....G.....T.A
Rh ACAGCCTCATGGGCCTCTCTCTCCCTTCACCCC*ATAGGACTTCAACAACCTCAATGAGGAAGACCAAACCATTCTGGTG 480
Ch .AG.....T.....C.C.....G.....GA.....A..
Ta CAG.....A.....TG....C.C..A.....T.A..CAG.....CG.....A..
Ma A.....T.A..CAG.....CGT.....A..
Rh GTAAGAGCTCAGCCC**********CTGGATCCCAATCCACTTCCTGCCTGGGTGACTTCAGCCATGTCATTCCAT 560
Ch .....**********G.....G.....
Ta .....TGCACTTGGCACAC....G.....T....CA.....A
Rh CTTGCCTAGCCTTGCTTTCTTCATCTGTAAAATAGGGTTAATAGCGCCTGTCTCAGTGGGACTGTTATGACAGTCAAATG 640
Ch ...A.....A...A.....T.....A.....
Ta ...AG.C.....G...T.....CT.....A...A.....T....GT.....A.....
Rh ACACAATGTGCATAACGTTCTGGCCCAGCATCTGGCACTTAAGGGTTCAATACGTGGCCACAGCCATGGCTATAAGAATG 720
Ch G..Y.....***.....A.....A.....T....
Ta .....A.....A.....C..A.....T...T....
Rh AAAATGACTTTTAAATTAGAAAATGAAAAGGCAGTTCTAGGTGAGGAATACTAAGGGCCTTAAGGTCAGTTTTTGGTAGG 800
Ch .....
Ta .....G.....A.....C..C..C.....C...C.....A.G..
Rh ACAGACTCTGGACTGACAGACGGAAATTTCCCCCACAGATGTGCCAGCTTCTTTTTCCCTTTAAAAAGTGAGTTTGTGTT 880
Ta G.C.....***..T..C.....GTG.....A.....C.....A...C.TA..CCC.....
Rh TTCCACCCTCATTGGTTGTGGCATTATCATTTACAGTTATTTTTCATGGTTCCAGTTGGTTGCAATGGTTCTCTCTCACG 960
Ta .....C..C...A.....T.....ACCA.....G.....C
Rh TTTGCATGACATTTCTTGGGTCAAACAAGAAGTGGAAGCCTTTGCTGAGTTCCTGGGCCATCTGTATTTGGGGCACTCAC 1040
Ta .....G.....C.....A...G.....G.C.G.....A.....
Rh TGCTCACATGCTGCACCCACTTCCATGTTGCGGTCTCTGTGATT 1084 1343 GCCTCCCAAGTTCAAGC 1360
Ta A.....T....T..... .T.T.....T

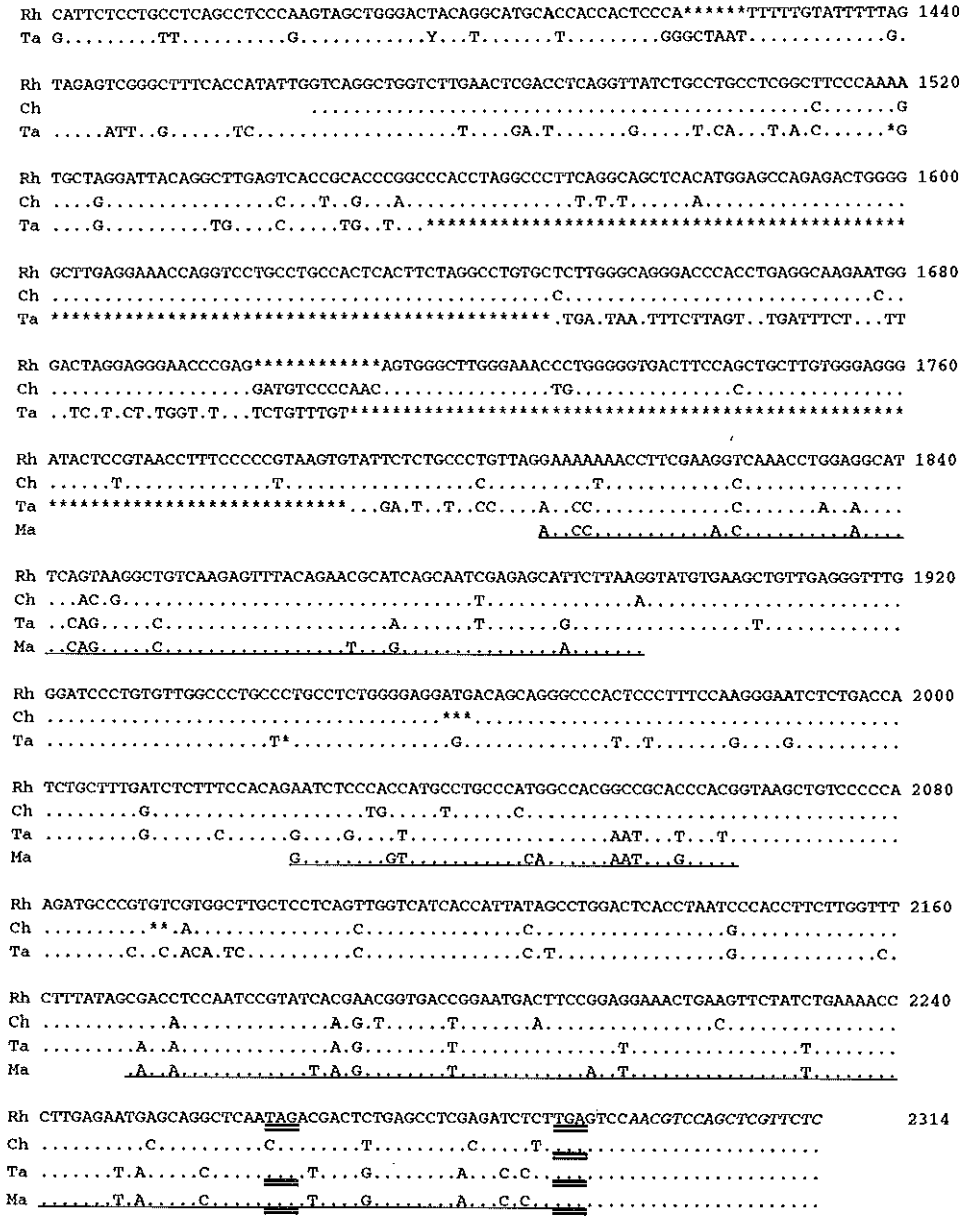


Fig. 6.2 DNA sequence alignment of primate IL-3 genes. Amplified fragments of chimpanzee (Ch), tamarin (Ta) and marmoset (Ma) IL-3 genes were cloned in the pTZ18R plasmid and sequenced in both orientations as described in chapter 2. Exon sequences were confirmed by analysis of cDNA clones. Multiple DNA sequence alignment was performed using

the "Clustal" algorithm (Higgins and Sharp, 1988) and included the RhIL-3 gene (chapter 4; accession number X51890). Identities are shown as dots and gaps are represented by asterisks. Spaces indicate nt sequences that were not determined. Coding sequences are underlined and termination sequences are double underlined. Primer sequences (2 and 7) used for DNA amplification are represented in italics. The newly identified primate IL-3 sequences are available under accession numbers X74875-X74879.

Sequence analysis of cDNA clones (retrieved by RT-PCR) derived from mRNA of COS cells confirmed their deduced exon structure (Fig. 6.2; Table 6.1) and did not reveal alternative RNA splicing in the IL-3 genes of chimpanzee, marmoset and tamarin. Moreover, the COS cell derived RT-PCR IL-3 fragments of chimpanzee, marmoset and tamarin (see Fig. 6.2) and rhesus monkey (see chapter 4) are apparently analogous to natural environmental splicing products as detected in man, gibbon and rodents. The complete nt sequences of the IL-3 coding regions of chimpanzee, tamarin and marmoset are shown in Fig. 6.2. The established sequences of the chimpanzee and tamarin IL-3 coding regions are based on relevant genomic fragments and confirmed by RT-PCR-retrieved cDNAs from COS cells, whereas the marmoset sequence is completely based on the RT-PCR cDNA products.

Western blot analysis of conditioned media from COS cells, transfected with genomic IL-3 expression constructs, revealed the production of chimpanzee, tamarin and marmoset IL-3 proteins which were readily detected by monoclonal antibodies (MCAs) directed against hIL-3 (Dorssers et al., 1991; Burger et al., 1994a). The immunoreactive IL-3 proteins were resolved in multiple higher molecular weight bands (±14-35 kDa), corresponding to variations in degree of glycosylation and the number of N-linked glycosylation sites. These results showed that functional IL-3 genes were amplified from chimpanzee, marmoset and tamarin. The biological activity of these gene products was assessed using a human IL-3 dependent AML-193 cell line as described in chapter 5 (see also section 2.2.2 of chapter 2). The chimpanzee IL-3 proteins excreted in the culture medium of COS cells strongly supported proliferation and DNA synthesis of AML-193 cells (Burger et al., 1994a). In contrast, neither tamarin nor marmoset IL-3 proteins induced detectable DNA synthesis in human AML-193 cells. These results, which are supported by preliminary studies using bacterially produced tamarin and marmoset IL-3, indicate that IL-3 proteins of these members of the Callitrichidae family are about 100 to 1000 fold less active than human and chimpanzee IL-3 in stimulating the proliferation of human hemopoietic cells. The amino acid changes in these proteins apparently interfere with the IL-3 signal transduction pathway, most likely due to impaired binding to the human heterodimeric IL-3 cell surface receptor.

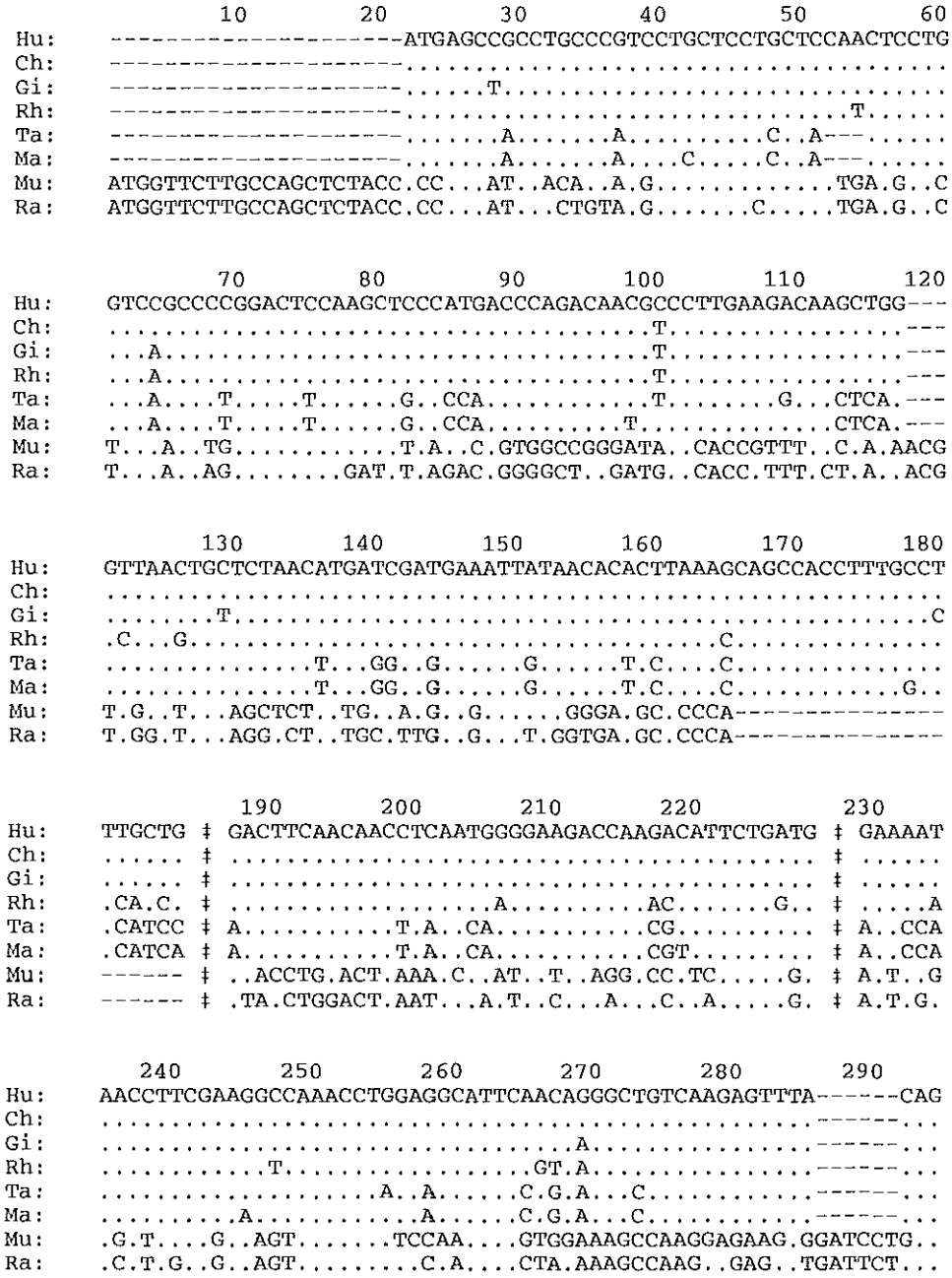
Table 6.1 Intron-exon boundaries of the identified primate and rodent IL-3 sequences[a]

	Intron I	Intron II
Hu	CTG **GT**GAGTA↔CCCAC**AG** GAC	ATG **GT**AAGAG↔CCGTT**AG** GAA
Ch	CTG **GT**GAGTA↔CCCAC**AG** GAC	ATG **GT**AAGAG↔CCGTT**AG** GAA
Rh	CCG **GT**GAGTA↔CCCAT**AG** GAC	GTG **GT**AAGAG↔CTGTT**AG** GAA
Ta	TCC **GT**GAGTA↔CCCAC**AG** AAC	ATG **GT**AAGAG↔CCCTT**AG** AAA
Mu	CCA **GT**GAGTA↔CTCAC**AG** GAA	AGG **GT**AAGAG↔CTCTT**AG** AAT
Ra	CCA **GT**GAGTA↔TCCTC**AG** GTA	AGG **GT**AAGAG↔CTCTT**AG** AAT
	Intron III	**Intron IV**
Hu	AAA **GT**ATGTG↔TCCAC**AG** AAT	ACG **GT**AAGCT↔TTTAT**AG** CGA
Ch	AAA **GT**ATGTG↔TCCAC**AG** AAT	ACG **GT**AAGCT↔TTTAT**AG** CGA
Rh	AAG **GT**ATGTG↔TCCAC**AG** AAT	ACG **GT**AAGCT↔TTTAT**AG** CGA
Ta	AAG **GT**ATGTG↔CCCAC**AG** GAT	ATG **GT**AAGCT↔TTTAT**AG** CAA
Mu	CAG **GT**GTGTG↔TCCAC**AG** AAA	GCG **GT**AAGCT↔CTCAC**AG** CTG
Ra	CAG **GT**GCGTG↔TCCAC**AG** AAA	GTG **GT**AAGCT↔GTCAC**AG** TTG

[a] The intron boundary sequences of the primate IL-3 genes of human (Hu), chimpanzee (Ch), rhesus monkey (Rh), tamarin (Ta), and rodent IL-3 genes of murine (Mu) and rat (Ra) with their single splice-donor dinucleotide (**GT**) and splice-acceptor dinucleotide (**AG**) and their 5' as well as 3' adjacent codons of the particular exons are indicated.

6.3 Multiple sequence alignment of the IL-3 coding regions and their corresponding proteins.

A multiple alignment, based on maximal homology between the IL-3 DNA and protein sequences of the various species, was established by the "Clustal" method of Higgins and Sharp (1988). This method is based on the iterative approach, i.e., the sequences are progressively aligned using the results of pairwise alignments (see section 2.4 of chapter 2). Interpretation of multiple protein alignments should be done cautiously since such methods don't take into account the genomic organization such as the intron/exon structure. The boundaries of the intron/exon junctions of the IL-3 genes of the various species appeared to be reasonably well conserved as shown in Table 6.1 and, therefore, codons could


10 20 30 40 50 60
Hu: ----------ATGAGCCGCCTGCCCGTCCTGCTCCTGCTCCAACTCCTG
Ch: ----------.....
Gi: ----------.....T.....
Rh: ----------.....T.....
Ta: ----------.....A.....A.....C..A---.....
Ma: ----------.....A.....A....C.....C..A---.....
Mu: ATGGTTCTTGCCAGCTCTACC.CC...AT..ACA..A.G.....TGA.G..C
Ra: ATGGTTCTTGCCAGCTCTACC.CC...AT...CTGTA.G.....C.....TGA.G..C
70 80 90 100 110 120
Hu: GTCCGCCCCGGACTCCAAGCTCCCATGACCCAGACAACGCCCTTGAAGACAAGCTGG---
Ch: .....T.....---
Gi: ...A.....T.....---
Rh: ...A.....T.....---
Ta: ...A....T.....T.....G..CCA.....T.....G...CTCA.---
Ma: ...A....T.....T.....G..CCA.....T.....CTCA.---
Mu: T...A..TG.....T.A..C.GTGGCCGGGATA..CACCGTTT..C.A.AACG
Ra: T...A..AG.....GAT.T.AGAC.GGGGCT..GATG..CACC.TTT.CT.A..ACG
130 140 150 160 170 180
Hu: GTTAACTGCTCTAACATGATCGATGAAATTATAACACACTTAAAGCAGCCACCTTTGCCT
Ch: .....
Gi: .....T.....C
Rh: .C...G.....C.....
Ta: .....T...GG..G.....G.....T.C....C.....
Ma: .....T...GG..G.....G.....T.C....C.....G..
Mu: T.G..T...AGCTCT..TG..A.G..G.....GGGA.GC.CCCA----------
Ra: T.GG.T...AGG.CT..TGC.TTG..G...T.GGTGA.GC.CCCA----------
190 200 210 220 230
Hu: TTGCTG ‡ GACTTCAACAACCTCAATGGGGAAGACCAAGACATTCTGATG ‡ GAAAAT
Ch: ..... ‡ ..... ‡ .....
Gi: ..... ‡ ..... ‡ .....
Rh: .CA.C. ‡ .....A.....AC.....G.. ‡ .....A
Ta: .CATCC ‡ A.....T.A..CA.....CG..... ‡ A..CCA
Ma: .CATCA ‡ A.....T.A..CA.....CGT..... ‡ A..CCA
Mu: ------ ‡ ..ACCTG.ACT.AAA.C..AT..T..AGG.CC.TC.....G. ‡ A.T..G
Ra: ------ ‡ .TA.CTGGACT.AAT...A.T..C...A...C..A.....G. ‡ A.T.G.
240 250 260 270 280 290
Hu: AACCTTCGAAGGCCAAACCTGGAGGCATTCAACAGGGCTGTCAAGAGTTTA------CAG
Ch: .....------...
Gi: .....A.....------...
Rh: .....T.....GT.A.....------...
Ta: .....A..A.....C.G.A...C.....------...
Ma: .....A.....A.....C.G.A...C.....------...
Mu: .G.T....G..AGT.....TCCAA....GTGGAAAGCCAAGGAGAAG.GGATCCTG..
Ra: .C.T.G..G..AGT.....C.A....CTA.AAAGCCAAG..GAG..TGATTCT...

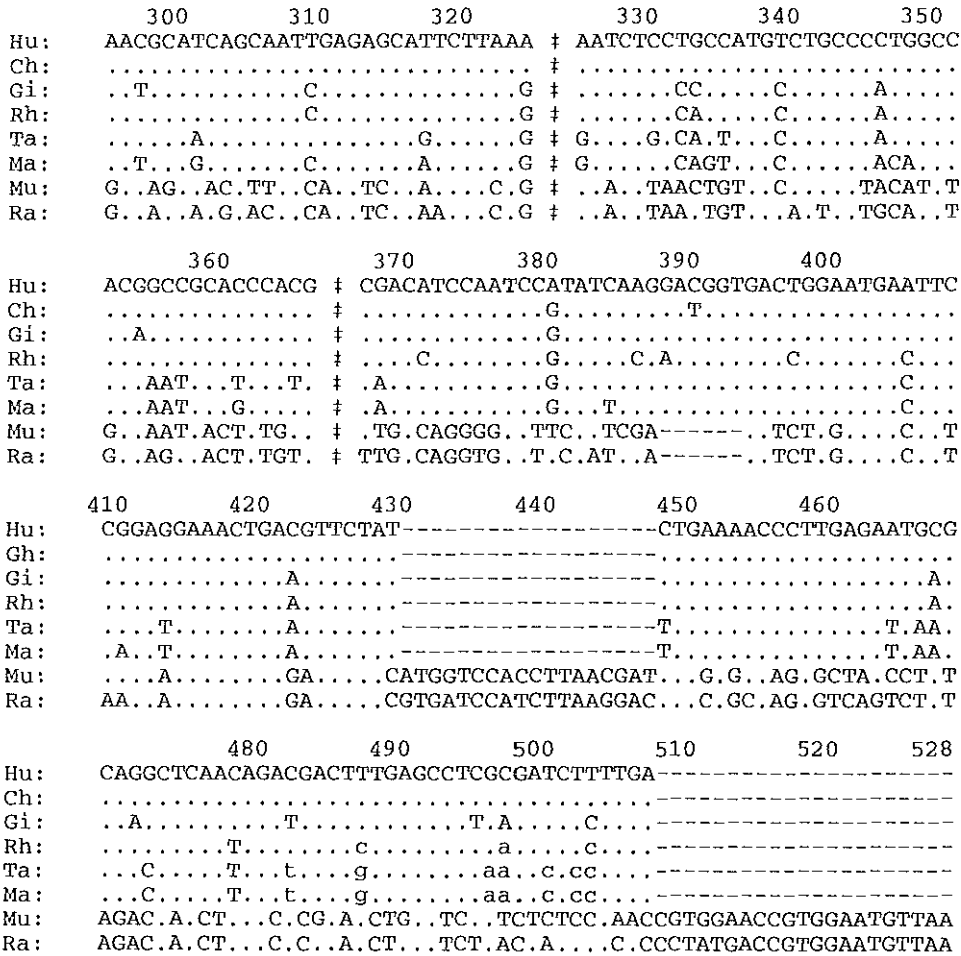


Fig. 6.3. Multiple sequence alignment of the IL-3 coding regions of human (Hu), chimpanzee (Ch), gibbon (Gi), rhesus monkey (Rh), tamarin (Ta), marmoset (Ma), murine (Mu) and rat (Ra). The complete nucleotide sequence of human IL-3 and the deviations from this sequence in the other species are presented by the one-letter nucleotide code, whereas identical nucleotides are indicated as dots. Gaps, introduced by the multiple sequence alignment to maximize the homology among the different species, are represented by horizontal lines (-). The genes of all identified IL-3 species comprise 5 exons (nt sequences of the coding regions are indicated in the multiple sequence alignment) and 4 introns (nt sequences are omitted). The exon-intron-exon junctions are indicated by ‡. Although the coding regions of the rhesus monkey and marmoset IL-3 genes are 27 nucleotides shorter due to the introduction of a termination codon, alignment of both sequences are given for the total length of the primate species with lower case nucleotides for deviations after the termination codons.

unambiguously be ascribed to a particular exon. Therefore, the conserved intron/exon junctions of IL-3 species were used to verify whether the computer-introduced gaps were located correctly. With this respect, the "Clustal" derived multiple alignment of the IL-3 proteins had to be adjusted to prevent alignment of 5 aa encoded by exon 2 of the rodent genes with residues encoded by exon 1 of the primates. This particular adjustment, which was based on the conserved regions around the IL-3 intron/exon junctions, could only be considered upon the identification of the genomic IL-3 sequences of man (Yang and Clark, 1987), mouse (Campbell et al., 1985), rat (Cohen et al., 1986), rhesus monkey (see chapter 4), tamarin and chimpanzee (see previous section). It is noteworthy that this correction (adjustment of the location of a computer-introduced gap) had no effect on the percent overall protein sequence homology. The "Clustal" derived protein alignment and conserved intron/exon junctions of the IL-3 genes (Table 6.1) served as basis for the multiple alignment construction of the IL-3 coding regions. The resulting multiple alignment of the DNA coding region and their corresponding IL-3 proteins are shown in Fig. 6.3 and 6.4, respectively.

The major differences between the newly identified IL-3 genes are briefly presented here. The nucleotide sequence of the chimpanzee IL-3 coding regions appeared to be virtually identical to the homologous sequences of the human gene (three changes corresponding to 99.3% sequence similarity; see Fig. 6.3). One of the deviations maps to the vexed nucleotide substitution at position 117 (see Fig. 3.10) which appeared to be polymorph in the human gene, i.e., C or T (Yang et al., 1986; Dorssers et al., 1987; Otsuka et al., 1988). The human and chimpanzee IL-3 proteins displayed only two differences, corresponding to 1.3 % divergence (Fig. 6.4). The established nt sequence of the tamarin and marmoset IL-3 coding regions are shown in Fig. 6.3. Notably, the tamarin and marmoset IL-3 sequences lacked the triplet encoding aa 11 of the signal peptide. The IL-3 genes of these simian primates encode mature proteins of 124 aa, actually identical in length to the RhIL-3 mature protein and nine residues shorter than the mature proteins of the higher primates (Fig. 6.3 and 6.4). Comparison of the coding regions of chimpanzee IL-3 with those of tamarin and marmoset revealed 72 deviations corresponding to 83% DNA sequence similarity and 81 deviations corresponding to 80.9% DNA sequence similarity, respectively. The IL-3 protein identity between chimpanzee and tamarin and between chimpanzee and marmoset was about 70%. The observed nucleotide and amino acid substitutions between the various species and the percent sequence divergence are summarized in Table 6.2. The similarity between the various primates was shown to vary from 80.9% (Ch/Ma) to 99.3% (Hu/Ch) for the DNA sequences and the protein identity varied from 69.2% (Rh/Ma) to 98.7% (Hu/Ch). Furthermore, somewhat lower but still significant similarity was found between the two rodent sequences (DNA: 78% and protein: 59%).In addition, pairwise analyses between primate and rodent IL-3 sequences showed rather low similarity which appeared to be less than 50% and less than 31% for

Table 6.2 Substitutions observed among present-day Il-3 coding regions and their corresponding proteins of various species[a]

Species compared	DNA			Protein		
	Substitutions	Gaps	Divergence	Substitutions	Gaps	Divergence
Hu/Ch	3/459	0	0.7	2/152	0	1.3
Hu/Gi	22/459	0	4.8	12/152	0	7.9
Hu/Rh	33/432	0	7.6	25/143	0	17.5
Hu/Ta	73/429	1	17.2	41/142	1	29.4
Hu/Ma	80/429	1	18.8	42/142	1	30.1
Hu/Mu	227/432	5	53.1	102/143	5	72.3
Hu/Ra	220/432	5	51.5	101/143	5	71.6
Ch/Gi	21/459	0	4.6	10/152	0	6.6
Ch/Rh	32/432	0	7.4	23/143	0	16.1
Ch/Ta	72/429	1	17.0	39/142	1	28.0
Ch/Ma	81/429	1	19.1	42/142	1	30.1
Ch/Mu	227/432	5	53.1	102/143	5	72.3
Ch/Ra	221/432	5	51.7	101/143	5	71.6
Gi/Rh	28/432	0	6.5	18/143	0	12.5
Gi/Ta	70/429	1	16.5	34/142	1	24.5
Gi/Ma	75/429	1	17.7	38/142	1	27.3
Gi/Mu	226/432	5	52.9	102/143	5	72.3
Gi/Ra	224/432	5	52.4	101/143	5	71.6
Rh/Ta	66/429	1	15.6	39/142	1	28.0
Rh/Ma	73/429	1	17.2	43/142	1	30.8
Rh/Mu	203/405	5	51.0	92/134	5	69.8
Rh/Ra	212/405	5	52.9	94/134	5	71.2
Ta/Ma	21/429	0	4.9	12/142	0	8.5
Ta/Mu	212/402	6	53.4	89/133	6	68.3
Ta/Ra	212/402	6	53.4	90/133	6	69.1
Ma/Mu	211/402	6	53.2	90/133	6	69.1
Ma/Ra	212/402	6	53.4	93/133	6	71.2
Mu/Ra	110/501	0	22.0	68/166	0	41.0

[a] Pairwise comparisons of IL-3 DNA coding regions and their corresponding proteins of human (Hu), chimpanzee (Ch), gibbon (Gi), rhesus monkey (Rh), tamarin (Ta), marmoset (Ma), murine (Mu) and rat (Ra). Substitutions considered in each pairwise comparison are presented as total number of substitutions per total common sites. Number of substitutions were calculated for the common regions in each pairwise comparative analysis and are expressed as actual number of substitutions per total common sites, i.e., bp or aa in case of DNA or protein sequences, respectively. Gaps (insertions and deletions) were counted as single events regardless of their length. Divergence is expressed as percentage and is equal to [substitutions + gaps]/[total sites + gaps] x 100.

```
                10        20        30        40        50
Hu :          MSRLPVLLLLQLLVRPGLQAPMTQTTPLKTSW-VNCSNMIDEI
Ch :          ..........................S.....-..........
Gi :          ..C...........S...........S.....-..........
Rh :          ..........H...S...........S.....-AK........
Ta :          ..H..I....-...S.....AP....S..ATQ-.....LRE..
Ma :          ..H..I....-...S.....AP...M....TQ-.....LRE..
Mu : MVLASSTT.IHTM....LM.FHL....SISGRDTHRLTRTL...SIVK..
Ra : MVLASSTT.I.CM..P.LM.FHQ...ISDRGSDAHHLLRTLD.RTIAL..
All:          S   *LL L *L*  GLQ *     * *       C  *  EI

                60        70        80        90        100
Hu : ITHLKQPPLPLLDFNNLNGEDQDILMENNLRRPNLEAFNRAVKSL--QNA
Ch : ..........................................------...
Gi : ..........................................K.....--...
Rh : ....N.....SP......E...T..V.K....S.....SK.....--...
Ta : V.L.N.....SSN.....R...R...KP..........QK.....--...
Ma : V.L.N....ASSN.....R...R...KP...K......QK.....--...
Mu : .GK.P-------EPELKTDDEGPS.RNKSF..V..SK.VESQGEVDPEDR
Ra : LVK.P-------VSGLN.SD.KAN.RNST...V..DE.TKSQEEFDSQDT
All: *  L                 **    L      R* NL  F          **

                110       120       130       140       150
Hu : SAIESILKNLLPGLPLATAAPTRHPIHIKDGDWNEFRRKLTFY------L
Ch : ........................R..............-------.
Gi : ..........P....M..........R.............K..------.
Rh : ..........P....M.......P..R.TN..R.D.....K..-------.
Ta : T....S..D.PL...M..N.SMQ...R.......D..M..K..-------.
Ma : A....N..D.PV...T..N.A.Q...R.......D.QM..K..-------.
Mu : YV.K.N.QK.NC...TSANDSALPGVF.R--.LDD..K..R..MVHLND.
Ra : TD.K.K.QK.KC.I.A.ASDSVLPGVYN.--.LDD.KK..R..VIHLKD.
All:   I S L* L  C*P **  *    **      D **F  KL FY      L

                160       170
Hu : KTLENAQAQQTTLSLAIF#
Ch : ..................#
Gi : .....E....M....E.S#
Rh : .....E...#.....E.S#
Ta : ....IK.P.#M.V..KTP#
Ma : ....IK.P.#M.V..KTP#
Mu : E.VLTSRPP.PASGSVSPNRGTVEC#
Ra : QPVSVSRPP.P.S.SDN.RPMTVEC#
All:  **     *    * *
```

Fig. 6.4. Alignment of the IL-3 proteins of human (Hu), chimpanzee (Ch), gibbon (Gi), rhesus monkey (Rh), tamarin (Ta), marmoset (Ma), murine (Mu) and rat (Ra). Computer assisted multiple sequence alignment was performed according to the "CLUSTAL" method of Higgins and Sharp (1989). Gaps, introduced by this method to maximize the homology among the different species, are shown by horizontal lines (-). One gap had to be relocated since an introduced gap in the rodent sequences resulted in the alignment of 5 aa encoded by exon 2 of the rodent genes with residues encoded by exon 1 of the primates. The aa sequence terminations are indicated by #.The complete aa sequence of human IL-3 along with differences in the other species are presented by the one-letter aa code, whereas identical residues are noted as dots. The line "All" shows the homology among all species with identical aa matches indicated by the corresponding one-letter symbol and conservative amino acid substitutions (ASTPG; NDBEQZ; HRK; MLIV; FYW) by asterisks. The five proposed helices for human IL-3 (Dorssers et al., 1991) are indicated as boxes.

DNA and protein, respectively.

Identical or functionally similar residues (conservative aa substitutions) in different IL-3 species may be important in view of the structure-function relationship. A consensus IL-3 protein sequence, indicated as "All" in Fig. 6.4, showed that in addition to the two cysteine residues present in hIL-3, only a few regions have been conserved during mammalian evolution. The best conserved regions correspond to the consensus residues 13-28 (signal peptide), 81-88, 103-118 and 132-143. The latter region contains a tyrosine (Y) residue (pos. 143) and two other aromatic phenylalanine (F) residues (pos. 136 and 142) which are conserved in all species. Another phenylalanine (F) at position 88 is also completely conserved and the one at position 64 is conserved in the primate species. However, it should be noted that several other phenylalanines present in the rodent IL-3 genes are not conserved at all. Analysis of substitution mutants of human IL-3 showed that alteration of highly conserved residues (e.g. pos. 70/71: ED→KR; pos. 81/82: RR→ED; pos. 135: E→K; pos. 142/143: FY→AT) results in a significant reduction (more than 3 logs) of biological activity (Dorssers et al., 1991). These results indicate that these conserved regions (at least several residues within them) indeed reflect important biological domains.

Secondary structure and three-dimensional architecture of proteins are known to be more conserved than their primary structure. Therefore, computer predicted secondary structures and hydrophobicity profiles may reveal more conformity among the various IL-3 species. The secondary protein structures (i.e., α-helix, β-plated sheet, random coil or β-turn) of the various IL-3 species were determined by combining several computer-assisted predictions (Chou and Fasman, 1978; Rose, 1978; Garnier et al., 1978). The five α-helices as proposed by Dorssers et al. (1991) for human IL-3 are indicated in Fig. 6.4 (helix I: 45-56; helix II: 69-81; helix III: 84-111; helix IV: 114-120; helix V: 134-156).

Analysis of the secondary protein structure predictions of the primate IL-3 species revealed that helices III-V are more or less conserved (the ends of the helices may differ somewhat among the IL-3 species), whereas helices I and II are not predicted for all primate IL-3 proteins. Analysis of rodent and primate

IL-3 species reveals one small conserved α-helical region (Fig. 6.4; pos. 84-92). Disruption of this proposed α-helix by introduction of helix-breaking residues strongly affects the biological activity of hIL-3 (Dorssers et al., 1991). In particular, substitution of residues 90 (R) and 91 (A) by Proline (P) and Glycine (G) respectively, resulted in at least 10,000-fold reduction of biological activity. Thus, the existence of such an α-helical region is strongly supported by the results of these mutant hIL-3 proteins.

The hydropathy profiles of the various IL-3 species (the Callitrichidae family is represented by the marmoset), which may reflect some three-dimensional structure properties of these proteins, are shown in Fig. 6.5. These profiles, showing the distribution of hydrophobic and hydrophilic residues along the protein chain, were constructed by calculating the mean hydropathy value of overlapping segments (in this case a window of six residues was used), according to the method of Kyte and Doolittle (1982). The highly hydrophobic part, present at the N-terminus of each IL-3 protein, indicates the leader-peptide which is processed during transport to the extracellular compartment. Furthermore, the hydropathy profiles of the primate IL-3 proteins show relevant similarities, which is also seen, although less clearly, for the two rodent sequences. However, the primate profiles share no significant homology with the rodent profiles. The latter suggests differential properties of the three-dimensional structures of primate and rodent IL-3 proteins.

6.4 Estimation of the number of synonymous and nonsynonymous substitutions

Pairwise comparison of the present-day IL-3 coding regions and their encoded proteins, as presented in Table 6.2, reveal in all cases that more than 40% of the observed DNA substitutions have resulted in amino acid substitutions of the protein chain. Comparative analysis of rhesus monkey sequences with other primate sequences showed an even higher divergence ranging from 59% (Rh/Ta) to 76% (Rh/Hu). The percent aa replacements per nt substitution for the Hu/Rh comparison was found to be particularly high (76%), which suggests an unusual high rate of evolution for the RhIL-3 protein. Notably, multiple nt substitutions within one codon, leading independently to an aa exchange, have been considered as only one aa substitution in these simple calculations. Thus, the number of substitutions observed among present-day IL-3 sequences are presumably an underestimation of the actual number of substitutions occurred since the radiation of these mammals. In addition, multiple substitutions at the same site (Kimura, 1980; Holmquist et al., 1982) may have occurred as well.
The method of Li et al. (1985), which includes corrections for those multiple hits (see above and section 2.4 of chapter 2), was used to calculate the number of synonymous (silent) and nonsynonymous (causing amino acid replacement) substitutions. Complete IL-3 coding regions as shown in Fig 6.3, which include

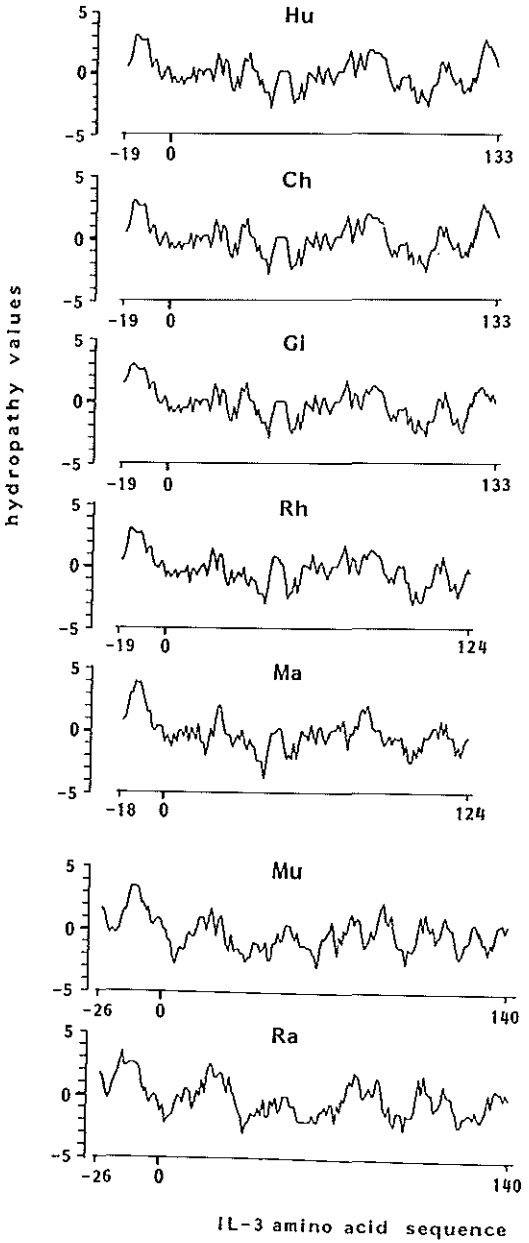


Fig. 6.5 Hydropathy plots of various IL-3 proteins. Hydropathy profiles., i.e.,the distribution of hydrophobic and hydrophilic residues along the protein chain of human (Hu), chimpanzee (Ch), gibbon (Gi), rhesus (Rh), marmoset (Ma), murine (Mu), and rat (Ra) were plotted according to the method of Kyte and Doolittle (1982). This algorithm calculates the mean hydropathy value of overlapping segments (a window of six residues was used). Positive values at the y-axis indicate hydrophobic regions, whereas negative values represent hydrophilic regions. The positive values at the x-axis refer to aa numbers in the mature IL-3 proteins (Hu, Ch, and Gi: 1-133; Rh and Ma: 1-124; Mu and Ra: 1-140). Negative values at the x-axis refer to aa numbers (relative to the processing site) of the signal peptide.

Table 6.3 Numbers of synonymous (K_S) and nonsynonymous (K_A) substitutions per site among the IL-3 coding regions of various mammalian orders[a].

Species compared	Percentage substitutions[b]		Ks/Ka (±SE)	Divergence[c] (%)
	K_S x 100 (±SE)	K_A x 100 (±SE)		
Hu/Ch	0.97 ± 0.98	0.57 ± 0.40	1.70 ± 2.09	0.66
Hu/Gi	10.50 ± 3.42	3.45 ± 1.01	3.04 ± 1.33	5.00
Hu/Rh	7.36 ± 2.91	8.72 ± 1.66	0.84 ± 0.37	8.41
Hu/Ta	23.34 ± 5.49	19.35 ± 2.66	1.21 ± 0.33	20.24
Hu/Ma	29.23 ± 6.45	20.39 ± 2.74	1.43 ± 0.37	22.36
Hu/Mu	139.96 ± 31.21	81.40 ± 8.80	1.72 ± 0.43	94.93
Hu/Ra	135.81 ± 29.04	74.83 ± 7.70	1.81 ± 0.43	88.31
Ch/Gi	11.61 ± 3.62	2.86 ± 0.91	4.06 ± 1.81	4.79
Ch/Rh	8.51 ± 3.16	8.08 ± 1.59	1.05 ± 0.44	8.18
Ch/Ta	24.58 ± 5.67	18.58 ± 2.59	1.32 ± 0.36	19.93
Ch/Ma	30.63 ± 6.66	20.41 ± 2.74	1.50 ± 0.38	22.69
Ch/Mu	139.37 ± 31.10	81.31 ± 8.73	1.71 ± 0.42	94.77
Ch/Ra	135.43 ± 29.19	75.74 ± 7.80	1.79 ± 0.43	88.98
Gi/Rh	10.70 ± 3.51	6.16 ± 1.37	1.74 ± 0.69	7.17
Gi/Ta	31.02 ± 6.68	15.52 ± 2.31	2.00 ± 0.52	18.91
Gi/Ma	30.78 ± 6.75	17.45 ± 2.47	1.76 ± 0.46	20.34
Gi/Mu	137.56 ± 33.14	81.43 ± 8.67	1.69 ± 0.44	94.08
Gi/Ra	136.90 ± 29.91	78.53 ± 8.11	1.74 ± 0.42	91.10
Rh/Ta	18.08 ± 4.66	17.53 ± 2.50	1.03 ± 0.30	17.65
Rh/Ma	20.20 ± 5.05	19.54 ± 2.67	1.03 ± 0.29	19.69
Rh/Mu	114.22 ± 22.95	81.30 ± 8.70	1.40 ± 0.32	88.95
Rh/Ra	122.04 ± 25.64	83.38 ± 8.88	1.46 ± 0.34	91.97
Ta/Ma	8.91 ± 3.11	3.64 ± 1.04	2.45 ± 1.10	4.80
Ta/Mu	160.47 ± 42.91	83.41 ± 8.98	1.92 ± 0.55	100.98
Ta/Ra	192.39 ± 54.08	76.84 ± 7.90	2.50 ± 0.75	102.02
Ma/Mu	158.50 ± 45.58	82.95 ± 8.77	1.91 ± 0.59	100.06
Ma/Ra	185.86 ± 56.41	78.00 ± 7.96	2.38 ± 0.76	101.34
Mu/Ra	21.60 ± 5.28	28.28 ± 3.24	0.76 ± 0.21	26.74

[a] Pairwise comparison of aligned IL-3 coding regions of human (Hu), chimpanzee (Ch), gibbon (Gi), rhesus monkey (Rh), tamarin (Ta), marmoset (Ma), murine (Mu), and rat (Ra). The number of substitutions per synonymous sites (K_S) and number of substitutions per nonsynonymous sites (K_A) were computed according to the method of Li et al. (1985).

[b] The number of substitutions and their standard errors are presented as percentage synonymous and nonsynonymous, i.e., number of substitutions per 100 synonymous sites (K_S x 100) and number of substitutions per 100 nonsynonymous sites (K_A x 100).

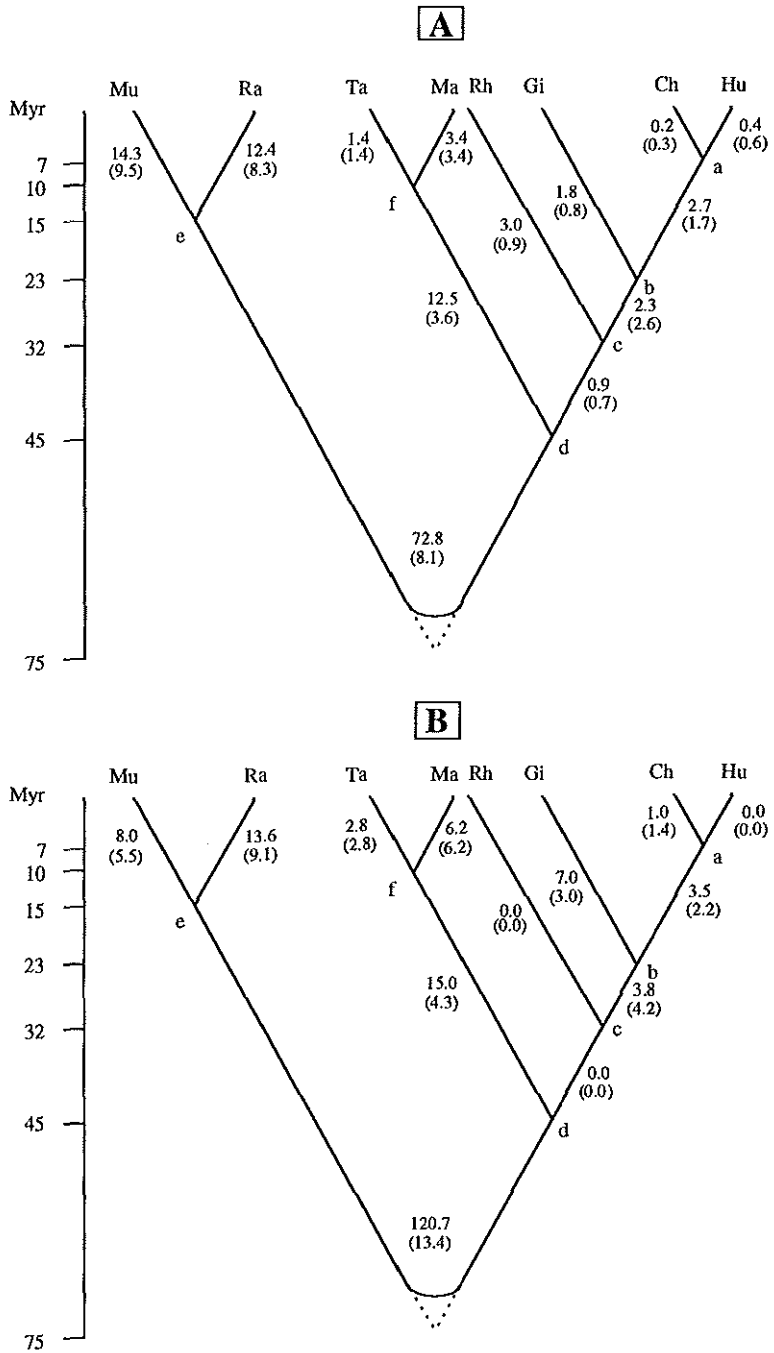
[c] Divergence was calculated and corrected according to the method of Li et al. (1985), i.e., $[K_S \times N_S] + [K_A \times N_A] / [N_S + N_A] \times 100$. N_S represents total number of synonymous sites, whereas N_A represents total number of nonsynonymous sites.

the 5' sequence encoding the leader peptide and the 3' 27 noncoding bases of exon 5 of the rhesus monkey, tamarin and marmoset genes (Fig. 6.4; pos. 478-504), were used to calculate the synonymous and nonsynonymous substitutions. Insertions or deletions (gaps) were omitted. The estimated number of substitutions per synonymous (K_S) and nonsynonymous (K_A) site and the K_S/K_A ratios of pairwise comparisons are listed in Table 6.3. The computed K_S/K_A ratios appeared to be much lower than the average value reported for functional genes (average $K_S/K_A = 5.3$; Li et al., 1985; Ticher and Graur, 1989). Notably, the studies of Li et al. (1985) and Ticher and Graur (1989) also revealed that rapidly evolving proteins show lower K_S/K_A ratios than more conserved proteins. Thus, the low K_S/K_A ratios as shown in Table 6.3 indicate that IL-3 belongs to the group of rapidly evolving proteins. Pairwise comparison of rhesus monkey and other primate taxa showed that the K_A value equals or exceeds the K_S value. These data suggest unequal rates of evolution among IL-3 species.

6.5 Construction of phylogenetic trees

Construction of phylogenetic trees from distance matrices are often used to find the correct tree topology and to estimate the branch lengths. Most methods to find a phylogeny are based on the average-linking clustering method, also known as the unweighted pair-group method with arithematic averages (UPGMA). If the rate of evolution among lineages is not constant, these UPG methods (procedures to construct phylogenies are reviewed by J.A.M. Leunissen, 1989) often lead to incorrect topologies. As alluded to earlier, the data presented in Table 6.3 suggest unequal rates of evolution of the IL-3 genes among different lineages. Therefore, phylogenies were constructed using the computer program FITCH (see section 2.4 of chapter 2) which does not assume constant evolutionary rates in branches and provides unrooted trees. The phylogenetic relationships of the different lineages, constructed from a distance matrix (sequence divergence data as shown in the right column of Table 6.3) based on the total number of substitutions per 100 sites calculated according to the method of Li et al. (1985), are shown in Fig. 6.6A. Construction of phylogenies from such an IL-3 distance matrix resulted in the established topology for the eight taxa used.

The actual substitution rates for each primate lineage can easily be calculated assuming that the times of divergence for Hu/Ch, Hu/Gi, Hu/Rh and Catarrhini/Platyrrhini are 7, 23, 32 and 45 million years (Myr), respectively (Pilbeam, 1984; Andrews, 1985). Furthermore, it was assumed that radiation of mammals occurred approximately 75 Myr ago and that the time of divergence between mouse and rat is 15 Myr (Li and Tanimura, 1987). The branching time of the genera of the Callitrichidae family, which consists of two major groups,


A
Mu
Ra
Ta
Ma
Rh
Gi
Ch
Hu
Myr
7
10
15
23
32
45
75
14.3
(9.5)
12.4
(8.3)
1.4
(1.4)
3.4
(3.4)
0.2
(0.3)
0.4
(0.6)
1.8
(0.8)
2.7
(1.7)
3.0
(0.9)
12.5
(3.6)
2.3
(2.6)
0.9
(0.7)
72.8
(8.1)
a
b
c
d
e
f
B
Mu
Ra
Ta
Ma
Rh
Gi
Ch
Hu
Myr
7
10
15
23
32
45
75
8.0
(5.5)
13.6
(9.1)
2.8
(2.8)
6.2
(6.2)
1.0
(1.4)
0.0
(0.0)
7.0
(3.0)
3.5
(2.2)
0.0
(0.0)
15.0
(4.3)
3.8
(4.2)
0.0
(0.0)
120.7
(13.4)
a
b
c
d
e
f

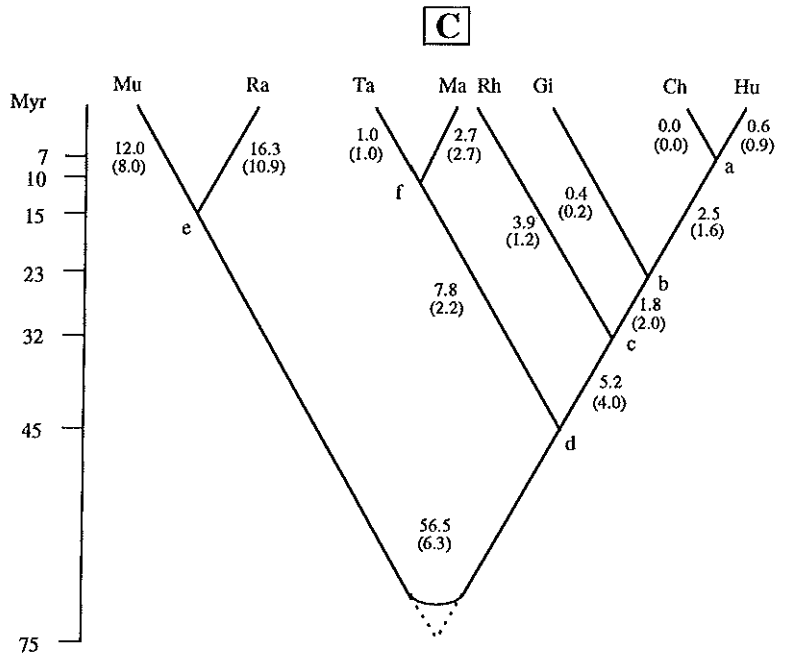


Fig. 6.6 Phylogenetic trees of IL-3 constructed from distance matrices. The phylogenetic relationships among the different lineages, i.e., human (Hu), chimpanzee (Ch), gibbon (Gi), rhesus monkey (Rh), tamarin (Ta), marmoset (Ma), murine (Mu) and rat (Ra) are shown with their times of divergence indicated on the scale by numbers of Myr. The number of substitutions per 100 sites (upper values) and their corresponding evolutionary rates expressed as number of substitutions per site per 10^9 year (values between brackets) and the ancestral nodes (a,b,c,d,e, and f) are indicated. The rates of nucleotide substitutions were calculated by implementing the estimated times of divergence. For example in Fig 6.3A, the rate of nucleotide substitution in the mouse lineage after the branching of the rat is: $(14.3/100)/(15 \times 10^6) = 9.5 \times 10^{-9}$ substitutions per site per year. The distribution of the total number of substitutions (corrected) and the synonymous and nonsynonymous substitutions among the different taxa are shown in panel **A, B and C,** respectively. All the substitution data were taken from Table 6.3.

i.e., tamarins and marmosets, has not been well established. The divergence between *Saguinus oedipus* (tamarin) and *Callithrix jacchus* (marmoset) was assumed to occur approximately 10 Myr ago. Rates of nucleotide substitutions were calculated by implementing the estimated times of divergence and are expressed as substitutions per site per 10^9 year (Fig. 6.6).

The phylogenies (Fig. 6.6) show consistently higher evolutionary rates in the IL-3 coding regions of rodents than of primates. The rates in the various Catarrhini decreased according to their branching order, i.e., rhesus monkey, gibbon and hominoids (chimpanzee and man). It should be noted that this method generates average rates for each lineage or segment between ancestral nodes and that the results depend on the accuracy of the divergence times. Furthermore, the low rate estimated for the segment between ancestral nodes "d" and "c" (Fig. 6.6A-C) has to be interpreted with caution since the use of rodent sequences as outgroup may cause the rate to be underestimated. In summary, it has been shown that the relative substitution rates of the studied IL-3 genes display a very large variation and that an apparent rate slowdown occurred for the IL-3 gene during hominoid evolution.

In addition to the construction of the phylogenetic tree from the corrected sequence divergence matrix, phylogenies were constructed using distance matrices generated from the synonymous and nonsynonymous substitution data presented in Table 6.3. The distribution of synonymous and nonsynonymous substitution rates among the different taxa are shown in Fig 6.6 panel B and C, respectively. Analysis of synonymous and nonsynonymous substitutions among the different taxa revealed a rather unusual distribution. The chimpanzee did not accumulate nonsynonymous substitutions during the 7 Myr of its divergence from the human branch which is reflected by the 98.7% IL-3 protein similarity between man and chimpanzee. Gibbon IL-3 did not acquire many nonsynonymous substitutions either and the synonymous substitution rate is about 15 times that of the nonsynonymous substitution rate. The most striking distribution was found in the rhesus monkey lineage following radiation of Old and New World monkeys (Fig. 6.6; split d), where exclusively nonsynonymous substitutions were detected. Thus, only nonsynonymous substitutions were fixed in the population leading to the rhesus monkey for a period of over 30 Myr. A progressive slowdown in nonsynonymous substitution rate was seen in the human lineage after the divergence of the Old and New World monkeys. However, the calculated rates of evolution in the primate species after their branching from the human lineage appeared to be lower than those of the corresponding human segments. For example, the human lineage accumulated $(1.8/100 + 2.5/100 + 0.6/100) / (32 \times 10^6) = 1.5$ substitutions per nonsynonymous site per 10^9 year compared to 1.2 substitutions per site per 10^9 year in the rhesus monkey. This difference is even more pronounced in the gibbon-vs-human lineage, i.e., 0.2 vs 1.3 substitutions per site per 10^9 year.

6.6 Summary and Discussion

The identification of the IL-3 coding sequences of chimpanzee and two members of the Callitrichidae family allowed analyses of relatively early as well as more recent events in primate IL-3 evolution. Multiple sequence alignments of

homologous IL-3 coding regions of eight different species and their encoded proteins enabled comparative evolutionary analyses. The alignment of primate and rodent IL-3 cDNAs and their encoded proteins, as presented in Fig. 6.3 and 6.4, slightly differ from those presented in chapter 3 (Fig. 3.8 and 3.10 and those previously presented by other investigators (Yang et al., 1986; Dorssers et al., 1987; Ihle, 1989). The IL-3 alignment presented in this chapter is in agreement with the conserved intron/exon structure.

Compared to IL-3 of other primates, tamarin, marmoset and rhesus monkey IL-3 lack nine C-terminal residues. Since it is rather unlikely that two identical evolutionary events occurred independently in the Callitrichidae and rhesus monkey lineages, we propose that the mutational event which resulted in an extension of the mature IL-3 protein of gibbon, chimpanzee and man occurred in between the branching of rhesus monkey and gibbon, respectively. Mutational analysis of hIL-3 demonstrated that this mutation is apparently of no functional significance (Dorssers et al., 1991).

The aligned homologous IL-3 proteins did not reveal much conformity among the various rodent and primate species as indicated by a low number of amino acid matches and in line with the species specificity pattern. Only a few small regions of IL-3 appeared to be preserved during evolution. In spite of differential lengths of rodent and primate IL-3 signal peptides, the hydrophobic (leucine-rich) region and the cleavage site are well conserved probably due to functional constraints. The two cysteine (Fig. 6.4; pos. 43 and 113), one tyrosine (pos. 143) and three phenylalanine (pos. 88, 136 and 142) residues present in the mature human IL-3 protein are completely conserved in all taxa and map within the essential biological domain of murine IL-3 (Clark-Lewis et al. 1988) and human IL-3 (Dorssers et al., 1991; Lokker et al., 1991a,b). The single disulphide bridge present in hIL-3 and the conserved aromatic residues probably represent important structural elements of the IL-3 architecture in agreement with highly conserved long range interactions of aromatic protons of the phenylalanine (pos. 142) in both human and rhesus monkey IL-3 (Dorssers et al. 1991). The three conserved regions (Fig. 6.4; 81-88; 103-118; and 132-143) present in the mature IL-3 protein map with proposed α helices in human IL-3 (Dorssers et al., 1991; Lokker et al., 1991a,b; Kaushansky et al., 1992). One small conserved α-helical region (Fig. 6.4 pos. 84-92) was predicted for all species. Disruption of such an α-helix by introduction of helix-breaking residues strongly affected the biological activity of hIL-3 (Dorssers et al., 1991). Mutational analysis of hIL-3 (Dorssers et al., 1991) showed that alterations of highly conserved residues are more deleterious for biological activity than substitutions at regions which were shown to be more variable. For instance, the hypervariable C-terminal as well as N-terminal regions of the mature IL-3 protein appeared to be of no functional significance in both human (Dorssers et al., 1991; Lokker et al., 1991a) and murine IL-3 (Clark-Lewis et al., 1988), whereas the conserved hydrophobic region (Fig. 6.4; residues 132-

143 of the consensus sequence) appeared to be essential for the biological activity of hIL-3 (Dorssers et al., 1991). It has been proposed that this conserved hydrophobic region is involved in receptor binding (Dorssers et al., 1991; Lokker et al., 1991a). In addition, it was shown that residues Pro^{33} and Leu^{34} (Fig. 6.4; pos. 60 and 61) play a critical role in modulating the biological activity of hIL-3 (Lokker et al., 1991a,b). Since the particularly proline-rich hydrophobic region from Pro^{30} through Leu^{35} (PPLPLL; Fig. 6.4 pos. 57-62) is completely deleted in the rodent IL-3 proteins and Leu^{34} of hIL-3 is exchanged by a serine [S] residue in the IL-3 sequences of tamarin, marmoset and rhesus monkey, it is likely that this region is involved in receptor binding and that differences in this hydrophobic region account for the impaired interspecies functional cross-reactivity.

Notably, tamarin IL-3 exhibited 70.6% protein sequence similarity with human IL-3 and was severely impaired (±900-fold) in supporting the proliferation of human IL-3 dependent cells (AML-193). In contrast, chimpanzee IL-3 displayed high aa sequence identity (98.7%) and was shown to be equally effective in supporting the proliferation of this human AML cell line (Burger et al., 1994a; see also section 6.2 of this chapter). Therefore, a panel of interspecies chimera between the chimpanzee and tamarin IL-3 proteins has been constructed and expressed in *Escherichia coli* and eukaryotic cells to investigate the role of substitutions in different protein domains on the functional species specificity (Dorssers et al., 1994). Analysis of the biological activity of the chimeric IL-3 variants indicated that the amino terminal region of IL-3 is indeed involved in the impaired interspecies functional cross-reactivity of orthologous IL-3 protein products. Substitutions at residues encoded by the first two exons (encompassing the "PPLPLL" region; Fig. 6.4 pos. 57-62) appear crucial in the functional species specificity, whereas C-terminal alterations show only moderate effects. Based on impaired interspecies functional cross-reactivity IL-3 of the chimeric IL-3 variants, comparison of aa sequences of chimpanzee and the New World monkeys, and disregarding conservative aa substitutions and substitutions also occurring in RhIL-3 (RhIL-3 is almost completely active on human cells), it was postulated that the major candidate residues likely to play an important role in the encountered species specificity are residues (see Fig. 6.4 for localization and substitutions of these residues) at position 47 (I→R), 53 (H→L), 69 (G→R), and 73 (D→R). Thus, the hIL-3 structure-activity relationship studies and analysis of chimeric IL-3 variants (Dorssers et al., 1994) indicate the presence of multiple receptor binding domains (Fig. 6.4; residues 47-73 and 132-143 of the consensus sequence) which is concordant with the heterodimeric nature of the IL-3 receptor. A similar multiple binding model has been hypothesized for GM-CSF (Shanafelt et al., 1991; Lopez et al., 1992), a related cytokine which mediates its action by binding to a cell surface receptor with significant sequence and structural resemblance to the IL-3 receptor (Bazan 1990). The functional

interspecies barrier of IL-3 suggests that either the binding site(s) on the growth factor has considerably diverged or that the heterodimeric IL-3 receptor itself has been subject to rapid evolution.

Pairwise comparison of present-day IL-3 DNA coding sequences of different taxa revealed that the extent of divergence among these species is similar to those estimated from cross hybridization of total single copy DNA and those estimated for orthologous noncoding flanking sequences, introns, exon silent sites, and pseudogenes (Miyamoto et al., 1988; Britten, 1986; Li and Tanimura, 1987; Goodman et al., 1990). Phylogenies constructed from IL-3 distance matrices showed that the various lineages accumulated nucleotide substitutions at widely different rates. These results are not consistent with the rate constancy assumption which is a major basis for the molecular evolutionary clock (Jukes, 1963; Kimura, 1968; and reviewed by Zuckerkandl, 1987). The differential rates of nucleotide substitutions among IL-3 species are supported by other studies reporting variable substitution rates among mammals (Britten, 1986; Li and Tanimura, 1987).

The total substitution rates observed in the primate IL-3 sequences (Fig. 6.6A) decelerated according to their branching order, while the rates in rodents are significantly higher than those estimated for primates. These results support the postulated rate-slowdown during hominoid evolution and are in line with the relatively high rates observed in rodents by other investigators (Goodman et al., 1971; Britten, 1986; Li and Tanimura, 1987; Seino et al., 1992). The latter was suggested to be primarily due to differences in generation time or, more precisely, in the number of germline DNA replications per time unit. Li and Tanimura (1987) reported that nuclear as well as mitochondrial human DNA sequences evolved slower than the corresponding sequences in monkeys suggesting a further rate-slowdown in the human lineage. Although the total substitution rate of the human IL-3 gene has become progressively slower after each separation of a primate lineage, it was in all cases higher than in the corresponding primate branch. Thus, a further rate-slowdown is apparently not a general phenomenon and variable rates are not exclusively attributed to differences in germline DNA replications. Changes in the efficiency of DNA repair mechanisms among species (Britten, 1986; Li et al., 1990), influencing the fixation rate of mutations, may be an important factor as well.

It has been suggested that differences in base composition (G + C content) among species could result in biased codon usage and differential substitution rates (Ticher and Graur, 1989). The existence of such a directional mutation pressure (towards a higher or lower G + C content) is a well-recognized phenomenon (Muto and Osawa, 1987; Sueoka, 1988; Saccone et al., 1989). A compartmentalized model of the nuclear genome in which the genes are distributed in compartments, the isochores, defined by there G + C content has been proposed (Bernardi et al., 1985, Bernardi and Bernardi, 1986). The G + C content at the third position of a codon presumably determines whether genes

belong to the same or different isochore. It has been suggested that only homologous gene pairs that belong to the same isochore are suited for evolutionary studies such as the determination of phylogenies and estimation of base substitution rates (Saccone et al., 1989). Homologous gene pairs that belong to the same isochore display rather universal substitution rates, whereas genes from different isochores show higher and more variable rates. The total G + C content of the IL-3 coding regions appeared to be 50% in all species and the G + C content at the third position of a codon was found to be about 62% for all primate species and about 56% for the rodent species. Therefore, the observed differences in substitution rate among IL-3 species are unlikely to be attributed to such a directional mutation pressure.

Assessment of the substitution rates at synonymous and nonsynonymous sites showed a rather unusual distribution in most IL-3 species. The level of synonymous substitutions is supposed to closely reflect the evolutionary divergence between homologous genes and is generally much higher than the level of nonsynonymous changes (Fitch, 1980; Li and Tanimura, 1987; Palumbi, 1989; Ticher and Graur, 1989). Figure 6.6B shows that the rate of nonsynonymous substitutions in IL-3 genes varies greatly among species. The premise that the rate of synonymous substitutions is similar among genes (Miyata et al., 1980) is not consistent with the calculated synonymous substitution rates of orthologous IL-3 genes in different mammalian orders which varied with about a factor of hundred (Fig. 6.6B). Differential synonymous substitution rates among genes have been demonstrated by several other investigators studying various genes (Li et al., 1985; Li and Tanimura, 1987; Ticher and Graur, 1989). Therefore divergence times between genes or species calculated from synonymous rates and thereupon used as a molecular clock should be interpreted with caution.

Analysis of K_S/K_A ratio's of IL-3 of the various primates revealed large differences in evolutionary behavior. The RhIL-3 gene did not accumulate synonymous substitutions in contrast to the gibbon gene which showed a clear preference for such silent substitutions. All the K_S/K_A ratios and especially those derived from rhesus monkey IL-3 comparisons are much lower than the reported average K_S/K_A ratio (Li et al., 1985). A study of Ticher and Graur (1989) showed that all rapidly evolving proteins (interferons, apolipoproteins, relaxin and *Thy*-1 antigen) have lower K_S/K_A ratios, i.e., 2 to 3, than the reported average value of Li et al. (1985), while more conservative proteins such as actin and tubilin gave much greater values (>100). Thus, the low K_S/K_A ratios for IL-3 species with emphasis on rhesus monkey indicate a rapid evolution of the IL-3 protein. Such an accelerated evolution in the rhesus monkey IL-3 gene may offer an explanation for the impaired interspecies functional cross-reactivity (see chapter 5; Burger et al., 1990a; Van Gils et al., 1994a). Furthermore, human/rat comparisons of 42 genes encoding functional

proteins (Ticher and Graur 1989) showed significantly lower values for K_A (highest reported value: 0.363 for apoliprotein A-II) and K_S (highest value: 1.074 for serum albumin) as those found for the IL-3 human/rat comparison (Table 3; K_A = 0.748 and K_S = 1.358). Thus, the rate of synonymous and especially nonsynonymous substitutions in IL-3 sequences of rodent and primate species are higher than those reported for other functional proteins so far. It remains to be elucidated as to whether the differences in K_S/K_A ratio and substitution rate correlate with distinct biological function of IL-3 among taxa.

As yet IL-3 appeared to be the most rapidly evolving hemopoietic growth factor. The high evolutionary rate of IL-3 is about equally fast as that found for pseudogenes. Such a rapid evolution may suggest that IL-3 is not subject to stringent functional constraints and that the majority of nucleotide changes are functionally neutral (or nearly neutral) mutations which are fixed in the population by random genetic drift. This concept is in line with the neutral theory of molecular evolution (Kimura, 1968) and asserts that the majority of changes, including those at nonsynonymous sites, are selectively neutral. One major corollary derived from this theory is that functionally less important proteins as well as less significant parts of a protein evolve faster than the more important ones. In view of the neutral theory, the rapid evolution of IL-3 denotes a trivial function or even a lack of function of the IL-3 protein. However, the highly conserved 160 bases long region upstream of the translation initiation of human, chimpanzee, rhesus monkey, tamarin, marmoset, rat and mouse IL-3 genes which encompasses the promoter and several other regulatory elements (see Fig. 6.2; Burger et al., 1994a) suggests that this promoter drives the expression of a functionally important and relevant gene. This IL-3 promoter region shows a somewhat higher sequence identity among species than the codon regions (e.g Ma/Ta: >99% vs 95.1%; Hu/Rh: 96% vs 92.4%; Hu/Ma: 88% vs 81.2%; Hu/Ra: 65% vs 48.5%). In addition, regulatory elements (ATTTA repetitive sequences) present in the 3' noncoding region of the Il-3 gene (see chapter 3 and 4) were conserved in human, mouse, rat and rhesus monkey. Inter-species comparison of available IL-3 introns revealed that the introns are slightly better conserved than the coding regions. Thus, the 5' and 3' noncoding regions as well as the IL-3 intron sequences are somewhat more conserved than the IL-3 coding regions indicating that adaptive evolutionary processes presumably have led to the large differences in aa sequences which in turn account for the unusual interspecies functional cross-reactivity pattern of IL-3. Furthermore, it is not likely that amino acids of a receptor binding protein are completely free to vary. The exclusive accumulation of nonsynonymous substitutions in rhesus monkey IL-3 after its branching from the other primates and the impaired inter-species binding (Burger et al., 1990a) further disagree with neutral fixation mechanisms. Pairwise comparisons between rhesus monkey IL-3 and other IL-3 species using the essential biological domain (Fig. 6.4; residue 43 through 143) revealed

K_S/K_A ratios similar to those found using the complete coding regions, whereas all other comparisons showed somewhat higher values emphasizing the singular evolutionary behavior of RhIL-3. Therefore, we favor the alternative hypothesis which proposes that mutational changes are fixed by positive darwinian selection with the most likely selective force being the structure of the IL-3 cell surface receptor.

Recent studies showed that high affinity IL-3 receptors require the coexpression and association of two distinct (α and β) subunits (Kitamura et al., 1991; Kitamura and Miyajima, 1992). In addition to the common β subunit (shared by IL-3, GM-CSF and IL-5), an IL-3 specific β subunit receptor molecule was identified in the mouse but not in the human genome which suggests that the heterodimeric IL-3 receptor differs in the human and mouse system (Kitamura et al., 1991a; Nicola and Metcalf, 1991; Miyajima, 1992). Apparently, the IL-3 molecule as well as its receptor underwent considerable changes during evolution after mammalian radiation. The key driving force may well have been the binding site of the β-subunit of the receptor, which, to conserve its functions during mammalian evolution, necessarily had to adapt to changes in, as so far known, three independently evolving growth factor proteins (IL-3, IL-5, and GM-CSF). This hypothesis is corroborated by the emergence, probably as a result of gene duplication, of an IL-3 specific β-subunit in mice, its fixation suggesting an adaptive advantage. Thus, a change of functional constraints may account for the numerous and exclusive fixation of nonsynonymous substitutions in the rhesus monkey IL-3 coding regions. Moreover, RhIL-3 may have been adapted to specific selectional pressure during its evolution, a phenomenon known as adaptive evolution. Such a selection of advantageous mutations as a mode of fixation has been suggested for several other proteins, including the cooperative sites of α and β hemoglobin chains during the emergence of heterotetrameric hemoglobin in the early jawed vertebrates (Goodman et al., 1975, 1987), active sites of serine protease inhibitors (Hill and Hastie, 1987), presumed active sites of ovomucoids (Laskowski et al., 1987), functional sites of bovine pancreatic trypsin inhibitor and its close homolog, spleen inhibitor (Creighton and Charles, 1987), langur stomach lysozyme (Stewart et al., 1987), baboon α-globin (Shaw et al., 1989), and ruminant placental lactogen (Wallis, 1993). It should be noted that the selectional evolutionary mechanism of the protease inhibitors is still under dispute (Graur and Li, 1988). More refined structural analysis of the IL-3 cell surface receptor and studies on the IL-3 structure/function relationships may provide data to further explain the unusual molecular evolution and interspecies functional cross-reactivity patterns of IL-3.

CHAPTER 7

GENERAL DISCUSSION

7.1 Human interleukin-3

The molecular cloning of human IL-3 (hIL-3), as described in chapter 3, enabled detailed analysis of its role in human hemopoiesis. The identification of hIL-3 genes has been also reported by others (Yang et al., 1986; Otsuka et al., 1988). In chapter 3, it was shown that the identification of the hIL-3 cDNA could be accomplished by virtue of hybridization with the 3' terminal region of the murine IL-3 cDNA. The IL-3 coding regions appeared to be extensively diverged (45% homology), whereas specific A + T-rich domains in the 3' noncoding region were highly conserved (93%). Multiple reiterations of a "ATTTA" motif are present in the IL-3 genes of various species as presented in this thesis (mouse, rhesus monkey, and man: see Fig. 3.8 and Fig. 4.5B) as well as in many other lymphokines and proto-oncogenes (Shaw and Kamen, 1986; Malter, 1989; Kruys et al., 1989; Bohjanen et al., 1991). These AT-rich sequences present in the 3' terminal region of these genes function as destabilizing elements that target their mRNAs for rapid cytoplasmic degradation. The presence of a functional hierarchy of "ATTTA" motifs in mediating rapid interleukin-3 mRNA decay has been demonstrated (Stoecklin et al., 1994), i.e., three adjacent motifs are required for binding of an inducible factor and are the recognition signal for mRNA degradation. IL-3 mRNAs are short-lived in unstimulated T cells and are stabilized by various stimulatory signals. Thus, the efficiency of IL-3 expression in T cells, the latter being the predominant natural source of this cytokine, is at least in part modulated by post-transcriptional mechanisms such as selective mRNA degradation. In addition, IL-3 expression is regulated by transcriptional activation as well (Mathey-Prevot et al., 1990; Shoemaker et al., 1990; Park et al., 1993). Several positive and negative regulatory elements, which were shown to be targets of potential transcription factors, are present in the promoter region of the IL-3 gene (see chapter 3 and 4). Recently, a novel critical regulatory site (IF-1: *in vivo* footprint-1) within the human IL-3 promoter region has been identified which is necessary, but not sufficient for IL-3 expression (Cameron et al., 1994). Osborne et al. (1995) identified a transcriptional enhancer, located 3 kb upstream of the human GM-CSF gene and conserved in mice, that is inducible by signals that mimic T cell receptor (TCR) activation and acts on the IL-3 promoter as well. Thus, the control of IL-3 expression occurs both at transcriptional and post-transcriptional level, mechanisms which ensure a tight regulation of the expression of this HGF. The complex regulation of IL-3 expression suggests that such a tight control is essential. Concordant with this concept is the relatively high conservation of the 5' and 3' noncoding regions, as compared to the protein coding sequences, among orthologous IL-3 genes (see chapter 3, 4 and 6). In addition, the introns display a significantly higher sequence homology than the exons (minus the 5' and 3' noncoding regions). Whether this indicates that the introns contain as yet unidentified regulatory

sequences has not been clarified.

The IL-3 gene has been mapped to the long arm of the human chromosome 5. The q23-32 region of human chromosome 5 encodes a cluster of HGFs that apart from IL-3, include GM-CSF, IL-4, IL-5, IL-9, the p40 subunit of IL-12, and IL-13. Analysis of the cytokine gene cluster on human chromosome 5 (near segment q31) and the syntenic region on mouse chromosome 11 revealed that the relative localization of the genes are similar and the order and direction of the HGF gene loci are determined as centromere → IL-13 → IL-4 → IL-5 → IL-3 → GM-CSF → IL-9 → telomere. Notably, IL-3 and GM-CSF are only 10 kb apart and both genes as well as the IL-4 gene are transcribed toward the centromere (reviewed by Zurawski and De Vries, 1994). The significance of the clustering of these HGFs in terms of gene regulation remains to be established. Whether the close proximity and similar structure of these genes indicate that they evolved by gene duplication events is not clear yet. The c-fms proto-oncogene which encodes the CSF-1 cell surface receptor is also located at 5q21-5q32 (Sherr, 1990). Initially, the GM-CSF gene and the c-fms proto-oncogene have been implicated in 5q deletions observed in specific myeloid disorders (Le Beau et al., 1986/1987). Now, it is apparent that many other HGFs may be involved in the pathogenesis of those myeloid proliferative disorders. Notably, patients with loss of a whole chromosome 5 or deletion of part of the long arm of this chromosome retain one normal chromosome 5. Whether loss of one allele could produce a gene-dosage effect that is by itself significant for the pathogenesis of such diseases, in the absence of a mutated second allele, is not clear. Moreover, myeloid proliferative disorders characterized by 5q deletions have not been proven to be the consequence of allelic loss of HGF genes.

Large-scale production of hIL-3 was accomplished by expression of the hIL-3 cDNA in *B. licheniformis*. (Van Leen et al., 1990/1991). The availability of large quantities of well-defined recombinant hIL-3 facilitated extensive (pre)clinical study of this HGF to determine its therapeutic window and side-effects. The pleiotropic effects of murine IL-3 observed both *in vitro* and *in vivo* suggests a broad spectrum of therapeutic applications for hIL-3. The potential clinical applications of recombinant hIL-3 include: 1) treatment of primary or chemotherapy-induced cytopenia, 2) acceleration of hemopoiesis upon bone marrow transplantation, 3) mobilization of hemopoietic stem cells into peripheral blood for harvesting purposes, 4) induction of differentiation and/or proliferation of malignant cells (leukemias and lymphomas) to make them more susceptible for cytotoxic agents, 5) enhancement of overall host defense in patients with immunodeficiency-related diseases (AIDS and cancer), 6) improvement of gene transfer to human hemopoietic stem cells. The (preliminary) results of phase I, phase II, and phase III clinical trials with recombinant hIL-3 are briefly summarized and discussed in section 4 of this chapter.

Analysis of the stimulatory *in vitro* activities of hIL-3 showed that this factor

stimulates immature blood cells to produce progeny along multiple blood cell lineages, including all major nucleated blood cell types as well as platelet and erythroid lineages and modulates the functional activity of monocytes and of eosinophilic and basophilic granulocytes (see chapter 5; reviewed by Wagemaker et al., 1990a; Ihle et al., 1992). Furthermore, hIL-3 has been shown to stimulate the *in vitro* proliferation of leukemic blast cells (see chapter 5; Dorssers et al., 1987; Delwel et al., 1987/1988; Löwenberg and Touw, 1993). The first clinical experience confirmed that hIL-3 stimulates the proliferation of human bone marrow progenitor cells (reviewed by Ganser et al., 1991; Gianella-Borradori, 1994). Thus, the *in vitro* and *in vivo* biological data as observed with hIL-3 are in concordance with those observed with murine IL-3.

Although IL-3 alone is capable of enhancing hemopoiesis, it is apparent from both *in vitro* and *in vivo* studies that combinations of cytokines provide the most effective mechanism for stimulating multilineage acceleration of hemopoiesis (reviewed by Moore, 1991; Ganser et al., 1991; Aglietta et al., 1993; Gianella-Borradori et al., 1994). Especially, *in vivo* combination therapy with a cocktail of GM-CSF and IL-3 were shown to act synergistically on multiple hemopoietic lineages. In this context, a genetically engineered human GM-CSF/IL-3 fusion protein (referred to as PIXY321) was developed to yield a single therapeutic agent (Curtis et al., 1991). On a weight-to-weight basis PIXY321 has a greater than additive biological activity (10- to 20-fold) in comparison to the individual cytokines from which it is composed (reviewed by Williams et al., 1991). The unanticipated enhanced biological activity of PIXY321 has to be attributed to the enhanced binding capacity of this fusion protein (as compared to IL-3) on target cells expressing GM-CSF and IL-3 receptors. Notably, the binding capacity of PIXY321 was shown to be approximately 10-fold enhanced as compared to IL-3 but was equivalent to that of GM-CSF. PIXY321 was shown to strongly stimulate the proliferation of multipotent CFU-GEMM and the more lineage restricted CFU-GM and BFU-E bone marrow progenitor cells both *in vitro* and *in vivo* and is currently being assessed in phase I/II clinical trials (Broxmeyer et al., 1995).

Other potent interleukin-3 receptor agonists with selectively enhanced hemopoietic activity as compared to recombinant hIl-3 have been developed (Lokker et al., 1991b; Lopez et al., 1992; Thomas et al., 1995). The most remarkable effect was seen with the IL-3 receptor agonist SC-55494, developed by Thomas et al. (1995) which showed a 60-fold increase in binding to the IL-3 receptor α subunit and a 20-fold greater affinity for binding to the heterodimeric α/β IL-3 receptor complex relative to recombinant hIL-3. The generation of these specific IL-3 receptor agonists, especially those that present selective enhancement of hemopoietic responses and concomitantly have limited impact on acute side effects, may have important therapeutic implications. Current (pre)clinical experience with recombinant hIL-3 indicates that the therapeutic efficacy of this HGF may be limited by side effects such as those

reported in mice (Wong et al., 1989), nonhuman primates (see next section; Van Gils et al., 1993b) and man (Biesma et. al., 1992; Denzlinger et al., 1993). At least some of these dose-limiting side effects are likely to be due to the release of inflammatory mediators such as sulfidoleukotrienes (Denzlinger et al., 1993) and histamine (Valent et al., 1989; Wagemaker et al., 1990b; Van Gils et al., 1993b/1995). Notably, SC-55494 showed a 5- to 10-fold enhanced hemopoietic response relative to its ability to activate the priming and release of inflammatory mediators. Therefore, such an IL-3 receptor agonist may have improved hemopoietic potency while dose-limiting inflammatory side effects are minimized. The differential activation of cellular proliferation versus inflammatory mediator release by IL-3 may indicate differential cell-specific IL-3 receptor expression or, alternatively, differential signaling coupled to distinct pathways such as the JAK/STAT and Ras signal transduction pathways.

In addition to IL-3 receptor agonists, the generation of specific antagonists (that can block the effects of IL-3) and generic antagonists (that can block simultaneously the effects of GM-CSF, IL-3, and IL-5) may have important therapeutic implications in situations such as allergic reactions. With respect to GM-CSF, a specific antagonist that is devoid of its biological activity but still binds the α-subunit of the GM-CSF receptor with normal affinity has been developed (Bagley et al., 1995). Characterization of sites of interaction between IL-3 and its receptor subunit(s) may permit the design of IL-3 specific antagonists as well.

7.2 Rhesus monkey interleukin-3 and species specificity of human interleukin-3

The molecular cloning of hIL-3 and its subsequent large scale production (see chapter 3) has prompted preclinical *in vivo* studies in human and nonhuman primates to identify its therapeutic window and side effects. Contrasting its broad range of action *in vitro*, administration of recombinant hIL-3 alone or in combination with other HGFs (GM-CSF, G-CSF, Epo and IL-1) to cynomolgus and rhesus monkeys showed only limited effects on the production of peripheral blood cells and circulating progenitors (Donahue et al., 1988; Mayer et al., 1989; Umemura et al., 1989; Krumwieh and Seiler, 1989; Geissler et al., 1990; Krumwieh et al., 1990: Monroy et al., 1991). Based on these preclinical results on hIL-3, it was postulated that the limited effects of hIL-3 in *Macaca* species should be attributed to its species specificity, i.e., impaired binding of hIL-3 to its cognate receptor expressed on cynomolgus and rhesus monkey cells. Hence, the rhesus monkey IL-3 was cloned and expressed in order to test this hypothesis and furthermore to enable preclinical studies with homologous IL-3.

The gene encoding RhIL-3 was isolated by screening a genomic rhesus monkey library with hIL-3 cDNA (see chapter 4). The DNA sequence of RhIL-3 displays 92.9% homology with the corresponding part of the hIL-3 gene and

their encoded proteins show 80.5% aa identity. Large-scale production of RhIL-3 by expression of RhIL-3 cDNA in *B. licheniformis* enabled *in vitro* and preclinical *in vivo* studies with homologous IL-3. It was shown that the isolated RhIL-3 gene encodes a functional protein that stimulated human as well as rhesus monkey hemopoietic cells (see chapter 5). However, RhIL-3 appeared to be approximately 100-fold more effective in stimulating rhesus monkey bone marrow cells than hIL-3 indicating an unidirectional species specificity. The diminished capacity of hIL-3 to support colony formation by rhesus monkey bone marrow progenitor cells was shown to reside in impaired binding of hIL-3 to the IL-3 cell surface receptor of rhesus monkey cells (Van Gils et al., 1994a). The established species specificity of hIL-3 implies that results from preclinical *in vivo* studies on hIL-3 in nonhuman primates suffered severely from the considerable species specificity and are not representative of the full potential of IL-3. Administration of homologous IL-3 to rhesus monkeys revealed a dose dependent production of bone marrow-derived blood cells, preceded by amplification of bone marrow hemopoietic progenitor cells (Wagemaker et al., 1990b; Van Gils et al., 1994a; Van Gils et al., 1995). Profound rises in basophilic, eosinophilic and neutrophilic granulocytes, monocytes, erythrocytes and platelets were manifested, while there was a moderate rise in the number of peripheral blood progenitor cells. The biological significance of the impaired binding of hIL-3 to rhesus monkey cells was demonstrated by direct *in vivo* comparison of the effects of high-dose recombinant human and rhesus monkey IL-3 (Van Gils et al., 1994a). Human IL-3, as compared to RhIL-3, elicited a relatively weak hemopoietic response in rhesus monkeys (see chapter 5: Fig 5.20) which is in concordance with the limited effects as observed by others (Donahue et al., 1988; Mayer et al., 1989; Umemura et al., 1989; Krumwieh and Seiler, 1989; Geissler et al., 1990; Krumwieh et al., 1990: Monroy et al., 1991).

The molecular cloning of the RhIL-3 gene and the ensuing availability of purified RhIl-3 protein allowed preclinical studies in rhesus monkeys to further identify the target cells and establish potential side effects to determine its clinical relevance which may increase our understanding of the IL-3 mediated hemopoietic and nonhemopoietic responses. With respect to side effects, IL-3 treatment of juvenile rhesus monkey elicits, apart from a strong stimulation of hemopoiesis (Wagemaker et al., 1990b), a dose- and time-dependent syndrome that includes urticaria, palpable lymph nodes, splenomegaly, thrombocytopenia, anemia, vomiting, diarrhea, intestinal bleeding, edema and arthritis (Van Gils et al., 1993b). The nature of the side effects, especially the high production of histamine producing basophils (Van Gils et al., 1995), indicates that IL-3 may be involved in the pathogenesis of acute type hypersensitivity reactions and arthritis. During administration of homologous nonglycosylated IL-3 to rhesus monkeys, reversal of hematologic effects as a consequence of neutralizing antibody production was manifested (Van Gils et al., 1994b). Analysis of

various IL-3 administration schedules in order to develop an optimal dose scheduling strategy revealed that recombinant nonglycosylated IL-3 is more effective by continuous i.v. infusion than by subcutaneous administration (Van Gils et al., 1993a).

Studies on IL-3 receptor expression on hemopoietic cells of rhesus monkeys showed that only a small proportion (2% to 5%) of rhesus monkey bone marrow cells stained specifically with biotinylated RhIL-3 (Wognum et al., 1995). Low expression of IL-3 receptors was seen at very early stages of hemopoiesis and IL-3 receptor expression continues to be low during differentiation into lineage-committed progenitors. IL-3 receptor expression gradually increases on precursors for B cells, granulocytes, monocytes, and erythrocytes, but finally declines to undetectable levels during terminal differentiation into mature peripheral blood cells, with the exception of basophils. The pattern of IL-3 receptor expression on rhesus monkey BM cells (Wognum et al., 1995) showed that IL-3 receptors are present (at low levels) on immature multipotent hemopoietic progenitor cells and that lineage-committed progenitors that differentiate into monocytes, neutrophils and basophils have the highest IL-3 receptor levels. The RhIL-3 receptor data are in line with the observed biological data, i.e., stimulation of the proliferation of immature multipotent BM progenitor cells and stimulation of the differentiation of lineage-committed progenitor cells. In addition, immature B cells were RhIL-3 receptor positive which is consistent with the effects of IL-3 on immature B cells of the mouse (Winkler et al., 1995) and man (Xia et al., 1992). This differential IL-3 receptor expression of distinct subsets of BM cells facilitates the isolation of subsets of specific progenitor cells. Analysis of IL-3 receptor expression on rhesus monkey BM and PB cells during the course of *in vivo* IL-3 treatment showed that the low IL-3 receptor expression on immature progenitor cells was not altered (Van Gils et al., 1995). The observed increase in IL-3 expression on BM and PB cells was shown to be predominantly attributable to the production of large numbers of basophils that express high levels of IL-3 receptors. These results indicate that IL-3, in addition to its direct effects on immature hemopoietic cells, may also stimulates hemopoiesis by indirect mechanisms such as induction of the release of inflammatory mediators (e.g. cytokines, leukotrienes and histamine) by accessory cells and other mature functional cells (e.g. basophils).

7.3 Molecular evolution and structure-function relationship of interleukin-3

The protein homology between murine and human IL-3 (28%) was shown to be considerably lower than that found for other cytokines (see chapter 3) indicating a rapid evolution of the IL-3 protein. Indeed, multiple sequence alignment and pairwise comparison of present-day IL-3 DNA and protein sequences of

different taxa (mouse, rat, marmoset, tamarin, rhesus monkey, gibbon, chimpanzee and man), as presented in chapter 6, revealed a relatively high accumulation of nt substitutions, especially at nonsynonymous sites, which resulted in low numbers of aa matches among IL-3 species. With respect to the protein homology, it was shown that only a few residues were conserved during mammalian evolution (see Fig. 6.4) which may explain the extreme species specificity, i.e., the impaired interspecies functional cross-reactivity (see chapters 5 and 6). Recently, the gene encoding ovine IL-3 has been identified and characterized (McInnes et al., 1993). The ovine IL-3 protein shares 30% and 36% protein identity with the mouse and human IL-3 protein, respectively. Even though a disulfide bridge had been shown to be essential for biological activity in murine and human IL-3 (Clark-Lewis et al., 1988; Dorssers et al., 1991), consistent with the conservation of two cysteine residues in all taxa (see Fig. 6.4: pos. 43 and 113), ovine IL-3 did not contain cysteine residues while it has the capacity to stimulate the proliferation and differentiation of ovine BM progenitor cells. Other conserved residues that appeared to be exchanged in ovine IL-3 are: GLQ $\rightarrow$ -LH (Fig. 6.4: pos. 24-26) which are part of the processing site, R $\rightarrow$ L (Fig. 6.4: pos. 81) and F $\rightarrow$ E (Fig. 6.4: pos. 142). Computer predictions showed that ovine IL-3 has the potential of forming a secondary structure similar to that of hIL-3 (McInnes et al., 1993), apparently in the absence of a disulfide bridge and the long range interactions of aromatic protons of the phenylalanine (Fig. 6.4: pos. 142). Thus, ovine IL-3 has highly diverged during its evolution while maintaining its biological activity on homologous cells which is in concordance with the concept that IL-3 is a very rapidly evolving HGF that, however, preserved its biological function.

Analysis of the distribution of substitution rates at synonymous and nonsynonymous sites in IL-3 coding regions showed that the K_S/K_A ratio's are significantly lower than those observed for most other functional genes, indicating a rapid evolution of the IL-3 protein (see chapter 6). Recently, the accuracy of methods for estimating the number of synonymous and nonsynonymous substitutions per site (including that developed by Li et al., 1985) were evaluated and subsequently new methods were developed (Ina, 1995) . With respect to the method of Li et al., 1985, it was shown that this method is favorable for the neutral theory of molecular evolution since it gives overestimates of the number of synonymous substitutions and underestimates of the number of nonsynonymous substitutions. Therefore, the rates of nonsynonymous substitutions as observed for IL-3 coding regions may actually even be higher as those presented in chapter 6 (Fig. 6.6C). Lower K_S/K_A ratio's and concomitantly higher rates of nonsynonymous substitutions further validated the hypothesis that mutational changes in IL-3 are fixed by positive darwinian selection with the most likely selective force being the structure of the heterodimeric IL-3 cell surface receptor. The β subunit of the IL-3 receptor necessarily had to adapt to changes in three independently evolving growth

factor proteins (IL-3, IL-5, and GM-CSF) in order to conserve its function during mammalian evolution. Notably, an IL-3 specific murine IL-3 receptor β subunit has been identified, whereas no human homologue has been detected (Kitamura et al., 1991a; Nicola and Metcalf, 1991). These data indicate a singular evolutionary behavior of the β subunit of the IL-3 receptor. In addition to changes in the ligand, the common β subunit had to adapt also to changes in the α subunit of the IL-3 receptor complex to form a high affinity receptor by association of the subunits. Notably, the α subunits of the human IL-3 and GM-CSF receptors are localized to the X-Y pseudoautosomal region (Gough et al., 1990; Milatovich et al., 1993). Mammalian sex chromosomes (X and Y) share a small terminal region of homologous DNA sequences, known as the pseudoautosomal region (PAR), which pair and recombine during male meiosis (reviewed by Marshall Graves, 1995). Genes from this PAR (as yet, only a dozen have been identified) are therefore inherited as autosomal genes, are present as two active copies in both males (X and Y chromosomes) and females (two X chromosomes), and escape inactivation of the X chromosome in females (Marshall Graves, 1995). The human pseudoautosomal IL-3 receptor α and GM-CSF receptor α genes were shown to be autosomal in mouse (chromosome 14 and 19, respectively (Milatovich et al., 1993). In addition, several other human pseudoautosomal genes within or near the PAR have pseudoautosomal counterparts in the great apes and monkeys, are autosomal in prosimians and detect no homologues at all in mouse. Thus, the gene contents of PARs in different mammalian species are poorly conserved and genes within this region have abundant hypervariable sequences (Milatovich et al., 1993) which suggest a peculiar molecular evolution of the IL-3 receptor α gene. Indeed, the overall sequence identity between hIL-3 receptor α and its murine counterpart was only 47% at the nt level and 30% at the aa level, respectively (Hara and Miyajima, 1992). These genetic data on the different subunits of the IL-3 receptor complex indicate that both IL-3 receptor subunits (α and β) may have underwent considerable changes during mammalian evolution which in turn may have influenced the molecular evolution and species specificity of IL-3. In summary, it is concluded that IL-3 and its receptor subunits have been under specific selectional pressure and that mutational changes have been fixed by positive darwinian selection to conserve specific functions and allow specific interactions between the ligand and its cognate receptor necessary for efficient IL-3 signaling.

7.4 Interleukin-3 and hemopoiesis

Considerable information has accumulated over the past several years relating the biological activities of IL-3 which support the concept that IL-3 has a unique capacity to support the proliferation and differentiation of a wide spectrum of hemopoietic cells (reviewed by Wagemaker et al., 1990; Ihle et al., 1992). The

pleiotropic activity of IL-3 has in turn elicited considerable enthusiasm for clinical trials of IL-3 in humans. Available for clinical trial since 1989, IL-3 has been administered to well over two thousand patients. In phase I-II clinical trials, the tolerability profile and the various activities have been defined, and ongoing phase III trials will finally establish its clinical relevance. Data derived from phase I-II studies including patients with advanced malignancies, secondary hemopoietic failure, myelodysplastic syndromes, aplastic anemia, and Diamond-Blackfan anemia indicate that IL-3 alone or in combination with more lineage-restricted and later acting HGFs has a moderate effect on thrombopoiesis, granulopoiesis and erythropoiesis in man, mainly by stimulation of early hemopoietic progenitor cells (reviewed by Ganser et al., 1991; Gianella-Borradori, 1994). The most promising and clinically important response to IL3 was the increase in platelet and reticulocyte counts. Furthermore, IL-3 alone or in combination with GM-CSF or G-CSF was shown to enhance neutrophil recovery during post-chemotherapy myelosuppression which warrant studies on dose-intensification of chemotherapy regimens. Other clinical situations, apart from chemotherapy or radiation induced pancytopenia (Ganser et al., 1990b; Ganser et al., 1991; Biesma et al., 1992; Postmus et al., 1992; Hoffman, 1993; Gianella-Borradori, 1994; Hovgaard and Nissen, 1995; Veldhuis et al., 1995; D'Hondt et al., 1995), in which IL-3 treatment (alone or in combination with other HGFs) was shown to produce some beneficial clinical effects include the area of peripheral blood progenitor cell mobilization (Brugger et al., 1993; Appelbaum, 1993). No or only a minor response of IL-3, which in many cases was limited by adverse effects, was seen in myelodysplastic syndromes (Ganser et al., 1990a; Kurzrock et al., 1991; Verbeek et al., 1995), Diamond-Blackfan anemia (Dunbar et al., 1991; Gillio et al., 1993; Bastion et al., 1995) and aplastic anemia (Ganser et al., 1990c). Furthermore, a major contribution from IL-3 in combination with GM-CSF or G-CSF may be expected in the area of sensitization of leukemia cells to cell cycle specific chemotherapeutic drugs (Tafuri et al., 1994) and in the area of treatment of hemopoietic genetic defects by gene transfer into hemopoietic cells (reviewed by Karlsson, 1991; Brenner, 1995).

Taken together the preliminary clinical data suggests that optimal doses of IL-3 in combination with other HGFs may mitigate or entirely eliminate the duration and severity of thrombocytopenia and decrease neutropenia after chemotherapy or BM transplantation. With respect to the effects of IL-3 on megakaryocytopoiesis and thrombocytopoiesis, it is not clear whether IL-3 directly exerts its effect on megakaryocytes and platelets or mediates its effect by induction of IL-6 production (Ganser et al., 1991). Nevertheless, it is conceivable that IL-3 will enter the clinic as a cytokine that has the capacity to reduce the incidence and severity of thrombocytopenia. Notably, several other factors including IL-6, IL-11, GM-CSF, SCF and LIF have proved to promote megakaryocytopoiesis at different stages of development and platelet production

(reviewed by Metcalf, 1994). The identification and characterization of thrombopoietin (TPO) in 1994 (see chapter 1; page 33 and 34) demonstrated that this factor has selective actions on megakaryocyte and thrombocyte proliferation and hence is the candidate regulator of circulating platelet levels. Indeed, preliminary preclinical data on thrombopoietin administered to mice (Lok et al., 1994; De Sauvage et al., 1994) and to normal nonhuman primates (Farese et al., 1995) showed that this factor stimulates megakaryocytopoiesis and platelet production *in vivo*. Kaushansky et al. (1995) provided evidence for the existence of two populations of megakaryocyte progenitor cells (CFU-Meg): one population that is responsive to IL-3 but requires TPO for full maturation and a second population that is absolutely dependent on TPO for both proliferation and differentiation. The data taken together indicate that TPO seems to be the prime candidate to treat patients with thrombocytopenia and that there may be a minor role for IL-3 in HGF combination therapy. Clinical trials on TPO will finally establish its value in clinical settings and define the role of IL-3 and other HGFs in megakaryocytopoiesis and thrombocytopoiesis.

The biological *in vitro* and *in vivo* data on IL-3, taken together, are consistent with the concept that IL-3 supports, either alone or in synergy with other factors (e.g. SCF, IL-6 or Flt-3/flk-2 ligand) the proliferation and differentiation of HPSCs and committed progenitor cells of multiple lineages including megakaryocytes, eosinophils, basophils, granulocytes, macrophages and erythroid progenitors. In each case, however, differentiation is also supported by other hemopoietic growth factors (e.g. GM-CSF supports differentiation of granulocytes/macrophages; TPO supports differentiation of megakaryocytes; IL-5 supports differentiation of eosinophils, IL-4 supports differentiation of mast cells; Epo supports differentiation of erythroid progenitors) indicating that IL-3 does not singularly control differentiation. Apparently, once committed to different lineages or shortly after commitment, cells seem to lose their ability to respond to IL-3 with the exception of basophils. A variety of data suggest that hemopoiesis is not absolutely dependent on IL-3. Thus, alternative factors must exist that can also support the proliferation and differentiation of hemopoietic stem cells. The lack of an essential role in constitutive hemopoiesis and the uniquely T cell origin of IL-3 have led to the concept that production of IL-3 has evolved within the immune system as a mechanism by which immunological reactions can broadly affect hemopoiesis (Ihle, 1992). Such a role would also predict that it will be possible to obtain mice in which the IL-3 gene has been deleted by homologous recombination. Unfortunately, the generation of such mice, which are essential to fully understand and define the role of endogenous IL-3 in hemopoiesis and especially the role it has in the development and content of immune responses, has not been reported yet. Alternatively, transgenic mice expressing antisense IL-3 RNA have been generated to downmodulate IL-3 production *in vivo*. Such mice were shown to exhibit either a B cell lymphoproliferative syndrome or

progressive neurologic dysfunction but did not exhibit hemopoietic abnormalities (Cockayne et al., 1994). The latter finding suggests that high levels of IL-3 are not required for normal hemopoiesis or it may even suggest that IL-3 is not an essential hemopoietic growth factor. The abnormal neurologic function of the antisense IL-3 transgenic mice suggest that IL-3 may play a role in the maintenance of normal neural processes which is consistent with the detection of IL-3 mRNA in normal astrocytes and microglial cells in the brain (Farrar et al., 1989). Furthermore, the B cell lymphoproliferative syndrome as observed in the antisense IL-3 transgenic mice is consistent to the concept that IL-3 plays an important role in B cell lymphopoiesis. Recently, it was shown that IL-3 nonresponsive mouse strains (A/J, AKR, RF, and NZB) carry an identical branch point deletion in the IL-3 receptor α subunit gene which results in aberrant splicing and hence cells from these mice do not express the IL-3 receptor α on their cell surface (Ichihara et al., 1995; Hara et al., 1995). The occurrence of several inbred mouse strains harboring the same genetic defect that abrogate IL-3 signalling and with apparently no impaired hemopoiesis further support the hypothesis that IL-3 is inessential for normal hemopoiesis. Notably, also GM-CSF deficient mice generated by gene targeting show no significant defect in hemopoiesis (Lieschke et al., 1994). Since the function of these HGFs overlaps, it may be that a defect in one HGF is compensated for by other HGFs with similar function. An appealing hypothesis is that pluripotent stem cells in the presence of IL-3 as a consequence of particular situations have a somewhat different outcome of a differentiation program than cells proliferating in the presence of factors that control constitutive hemopoiesis. Whether such particular situations (e.g. allergic reactions) exist, where IL-3 is absolutely required, is as yet not clear. It can be concluded that although considerable information relating to the biological activities of IL-3 has accumulated over the past several years, especially since the molecular identification and the ensuing availability of several IL-3 species, the effects of IL-3 on normal hemopoiesis are somewhat controversial and its physiological role is not clearly defined yet.

SUMMARY

Blood cell production, or hemopoiesis, is maintained from a small, persistent pool of pluripotent hemopoietic stem cells (PHSCs). PHSCs are defined by the ability of self-renewal as well as the capacity to give rise to progenitor cells which proliferate and differentiate into functional peripheral blood cells. The PHSC compartment is capable of producing an entire functioning hemopoietic system which itself can produce new PHSCs. Effective hemopoiesis requires tight control provided by positive and negative regulatory signals.

The major control mechanism of hemopoiesis is mediated by a number of glycoproteins designated as hemopoietic growth factors (HGFs). In addition, more aspecifically acting molecules such as steroids, prostaglandins, and a number of other cytokines as well as microenvironmental signals have also been shown to be involved in the regulation of hemopoiesis. An outline on the regulation of hemopoiesis and the genetic, biochemical, biological and physiological features of the major HGFs are given in chapter 1.

Interleukin-3 (IL-3) was originally thought to be a primary regulator of stem cell proliferation. In mice, the factor had been recognized and studied *in vitro* under a variety of designations, including stem cell activating factor (SAF) and multilineage-colony-stimulating factor (multi-CSF). Initially, it was characterized from partially purified conditioned medium of a myeloid leukemia cell line. The molecular cloning of murine IL-3 (mIL-3) enabled much more refined basic studies to elucidate its physiological role in hemopoiesis. From these initial studies it became clear that IL-3 supports the proliferation and differentiation of a variety of hemopoietic progenitors and affects the function of terminally differentiated cells of several lineages. Consistent with these findings, mIL-3 administered to mice greatly expanded hemopoiesis *in vivo*. These initial studies suggested that IL-3, in contrast to many other HGFs, is an early and broad-acting HGF as it stimulated multipotent hemopoietic cells and thereby the production of a variety of cell types. The pleiotropic effects of mIL-3 observed both *in vitro* and *in vivo* suggested a broad spectrum of therapeutic applications for the homologous human factor.

The experiments described in this thesis were aimed at the identification of human IL-3. The molecular cloning of the gene encoding human IL-3 (hIL-3) is presented in chapter 3. The identification was established by virtue of hybridization of the gene with the 3'-terminal region of the murine IL-3 cDNA. The highly conserved 3'-untranslated region displays 93% sequence homology

with the murine counterpart, whereas the coding regions share only 45 % homology. The hIL-3 gene appeared to be located on chromosome 5 in the proximity of a cluster of other HGF genes. The isolated hIL-3 cDNA encodes a precursor protein of 152 aa which following processing yields a mature protein of 133 aa. The hIL-3 protein displays only 29% homology with mIL-3, which is significantly lower than that found for other HGFs. Recombinant hIL-3 has been successfully produced by expression of the isolated cDNA in cells of various organisms, including eukaryotic, prokaryotic and yeast cells. The expression of LacZ/hIL-3 fusion proteins facilitated the production of antibodies directed against the hIL-3 protein. Large-scale production of the hIL-3 polypeptide was enabled by expression of the hIL-3 cDNA in the bacterial host, *Bacillus licheniformis* (*B. licheniformis*). The ensuing purification of hIL-3 to homogeneity facilitated detailed biological characterization of this HGF and analysis of its role in hemopoiesis.

The *in vitro* effects of hIL-3 on various hemopoietic cell populations, including normal human bone marrow cells as well as malignant hemopoietic cells and cell lines, are described in chapter 5. Studies of normal bone marrow cells demonstrated that hIL-3, irrespective of the type of expression system and its host organism, stimulates the proliferation of multipotent human bone marrow progenitor cells and supports the production of bone marrow derived precursors committed to all myeloid-derived lineages. Furthermore, it was shown that hIL-3, in many cases, strongly stimulates the in vitro proliferation of human leukemic blast cells. Human IL-3 exerted its action at a concentration of approximately 5 x 10^{-11} mol per liter. The *in vitro* biological characterization of hIL-3 demonstrated that the spectrum of biological activities of hIL-3 is similar to that observed for mIL-3 on murine cells. Therefore, hIL-3 resembles mIL-3 with respect to its genetic as well as its biological properties.

The large-scale production of homogeneous hIL-3, as described in chapter 3, prompted preclinical *in vivo* studies in nonhuman primates such as the *Macaca* species to identify the therapeutic window of IL-3. Contrasting its broad range of action *in vitro*, recombinant hIL-3 administered to rhesus monkeys and cynomolgus monkeys exerted only limited effects on the production of peripheral blood cells. To test the hypothesis that these limited effects of hIL-3 should at least be partly attributed to its species specificity (impaired interspecies functional cross-reactivity), it was decided to clone the gene encoding rhesus monkey IL-3 (RhIL-3). The isolation of the RhIL-3 gene and construction of a RhIL-3 cDNA are described in chapter 4. The nucleotide sequence of the RhIL-3 gene displays about 93% homology with the corresponding part of the hIL-3 gene and the genes showed an identical genomic organization. Potential regulatory elements identified in the IL-3 genes of mouse, rat and man were also found in the RhIL-3 gene indicating a common mechanism of regulation of IL-3 expression in these species. The isolated RhIL-3 gene encodes a 143 aa precursor polypeptide, nine residues shorter than the

human protein. Protein identity between human and RhIl-3 was found to 89.5% for the signal peptide (19 aa) and 80.5% for the mature protein (124 aa). After expression of a genomic fragment in COS cells, RhIL-3 cDNA was constructed, which enabled production of the RhIL-3 protein. Expression of a RhIL-3 cDNA in *B. licheniformis* and purification of the RhIL-3 protein was performed analogous to the expression and purification of hIL-3.

Biological data presented in chapter 5 demonstrated that the constructed RhIL-3 cDNA encodes a functional protein as measured by the capacity to stimulate human and rhesus monkey hemopoietic cells. Comparison of the biological activities between hIL-3 and RhIL-3 assessed on human and rhesus monkey bone marrow cells revealed differential stimulatory activity, especially on rhesus monkey bone marrow cells. RhIL-3 appeared to be approximately 100-fold more effective in stimulating rhesus monkey hemopoietic progenitors than hIL-3, whereas RhIL-3 and hIL-3 showed comparable stimulatory activity on normal as well as malignant human hemopoietic cells. Thus, these comparative studies showed a uni-directional species specificity of hIL-3 which explains the limited effects of hIL-3 observed in preclinical studies administering hIL-3 to Macaca species. An impaired binding of hIL-3 to its cell surface receptor on rhesus monkey cells accounted for the observed functional species specificity. Furthermore, administration of hIL-3 and RhIL-3 to rhesus monkeys convincingly demonstrated that preclinical studies with hIL-3 in nonhuman primates have considerably suffered from the species specificity of hIL-3 and are therefore not representative for the full potential of hIL-3.

To allow analysis of relatively early as well as more recent events in primate IL-3 evolution, the identification of the IL-3 coding sequences of chimpanzee, marmoset and tamarin was essential (chapter 6). Chimpanzee IL-3 displays 98.7% protein identity to hIL-3, whereas the IL-3 species of marmoset and tamarin show approximately 70% protein identity to hIL-3. Similar to RhIL-3, marmoset and tamarin IL-3 lack nine C-terminal residues. Chimpanzee and human IL-3 were shown to be equally effective in stimulating the proliferation of hIL-3 dependent AML-193 cells, whereas tamarin IL-3 was considerably less active than hIL-3 (±900-fold) in supporting the proliferation of these human cells. Multiple alignment of the IL-3 coding sequences of mouse, rat, marmoset, tamarin, rhesus monkey, gibbon, chimpanzee and man showed that only a few regions have been conserved during mammalian evolution. Analysis of hIL-3 muteins, natural IL-3 variants of other species and a panel of interspecies chimera between chimpanzee and tamarin allowed for the identification of functional domains of IL-3 (chapter 6). These studies indicated the presence of at least two receptor binding domains (Fig. 6.4: residues 47-53 and residues 132-143).

Phylogenetic trees constructed from distance matrices of IL-3 coding sequences showed large differences in IL-3 evolution rate. The accumulation of nucleotide substitutions in rodents occurred at a significantly higher rate than in

primates. Furthermore, a slowdown of the rate of hominoid evolution was evident. Analysis of the substitution rates at synonymous (silent mutations) and nosynonymous (resulting in aa change) sites revealed a rather unusual distribution in most IL-3 species. Extremes were rhesus monkey IL-3, which accumulated few synonymous substitutions, and gibbon IL-3, which had accumulated almost exclusively synonymous substitutions. In rhesus monkey IL-3, nonsynonymous substitutions outnumbered synonymous substitutions, which could not be readily explained by a random substitution process. Therefore, it was postulated that during evolution of IL-3, the majority of amino acid replacements and the impaired interspecies functional cross-reactivity originate from selection mechanisms with the most likely selective force being the structure of the heterodimeric IL-3 cell surface receptor. The key driving force may well have been the binding site of the β subunit of the IL-3 receptor, which, to conserve its function during mammalian evolution (binding of growth factor ligands and association with α subunits to result in appropriate signal transduction), necessarily had to adapt to changes in three independently evolving growth factor proteins (IL-3, IL-5, and GM-CSF) as well as evolutionary changes in the α-subunit of the IL-3 receptor complex. The singular evolutionary behavior of the RhIL-3 gene (numerous and exclusive fixation of nonsynonymous substitutions) may offer an explanation for the impaired binding of the hIL-3 to its cognate cell surface receptor on rhesus monkey cells.

SAMENVATTING

Bloedcel-produktie (hemopoiese) wordt in stand gehouden door een klein aantal pluripotente hemopoietische stamcellen (PHSCs). Deze stamcellen hebben de mogelijkheid om dochtercellen te produceren die alle eigenschappen van stamcellen hebben behouden ("self-renewal") en tevens het vermogen om te differentiëren in de verschillende typen perifere bloedcellen. Stamcellen zorgen dus voor de aanmaak van alle bloedcellen en waarborgen door het principe van "self-renewal" de continuïteit van de bloedcelvorming. Een goed functionerende bloedcelvorming vereist een nauwkeurige balans tussen positieve en negatieve regulatie signalen.

Een aantal glycoproteïnes die bekend staan als hemopoietische groeifactoren (HGFs) reguleren in belangrijke mate de aanmaak van bloedcellen. Naast de HGFs zijn een aantal andere, minder specifiek functionerende moleculen zoals steroiden, prostaglandinen en andere cytokinen betrokken bij de regulatie van de bloedcel-produktie. Tevens speelt de hemopietische micro-omgeving een belangrijke rol. In hoofdstuk 1 worden de hoofdlijnen van de regulatie van de bloedcelvorming en de genetische, biochemische, biologische en fysiologische kenmerken van de belangrijkste HGFs behandeld.

Interleukine-3 (IL-3) werd oorspronkelijk beschouwd als een primaire regulator van stamcelproliferatie. Bij muizen was de factor geïdentificeerd en bestudeerd onder de termen "stamcel activerende factor" (SAF) en "multilineage-kolonie-stimulerende factor" (multi-CSF) en gedeeltelijk gezuiverd uit het supernatant van een myeloide leukemische cellijn. Uitgebreide studies gericht op de fysiologische rol van IL-3 werden mogelijk gemaakt door de klonering van het gen voor IL-3 van de muis. Deze studies lieten zien dat IL-3 de proliferatie en differentiatie van een groot aantal hemopoietische voorlopercellen stimuleert en dat deze factor tevens de functie van verschillende perifere bloedcellen moduleert. In overeenstemming met deze *in vitro* studies met het IL-3 van de muis werd er een verhoogde hemopoietische activiteit waargenomen in IL-3 behandelde muizen. De studies wezen erop dat IL-3, in tegenstelling tot de meeste andere HGFs, in staat is om de bloedcel-produktie te stimuleren langs meerdere hemopoietische differentiatie-lijnen. De brede werking van mIL-3 wijst op een groot scala van therapeutische toepassingen voor het menselijke IL-3.

De experimenten beschreven in dit proefschrift waren gericht op de identificatie van het menselijke IL-3. Hoofdstuk 3 beschrijft het kloneren van

het menselijke IL-3, m.b.v. recombinant DNA technieken. De identificatie van het menselijke IL-3 gen was tot stand gekomen m.b.v. hybridisatie technieken waarbij gebruik werd gemaakt van het 3' niet coderende deel van het mIL-3 cDNA. Dit sterk geconserveerde gebied vertoont 93% homologie tussen muis en mens. Daarentegen vertonen de coderende gebieden slechts 45% homologie. Het menselijke IL-3 gen bleek gesitueerd op chromosoom 5, temidden van een cluster van andere HGF genen. Het menselijke IL-3 cDNA codeert voor een 152 aminozuren lang precursor eiwit dat uitgescheiden wordt als een matuur eiwit van 133 aminozuren. Het menselijke IL-3 eiwit vertoont slechts 29% homologie met het IL-3 van de muis, hetgeen beduidend lager is dan voor andere HGFs. Het menselijke IL-3 cDNA is in verscheidene organismen tot expressie gebracht en het IL-3 eiwit is ondermeer in eukaryote, prokaryote en gist cellen geproduceerd. De produktie op grote schaal van recombinant menselijk IL-3 werd bereikt door het cDNA tot expressie te brengen in een bacteriële gastheer (*Bacillus licheniformis*). De volledige zuivering van het IL-3 eiwit maakte onderzoek naar de rol van IL-3 in de regulatie van de bloedcelvorming mogelijk.

Hoofdstuk 5 beschrijft de *in vitro* effecten van het menselijke IL-3 op verschillende populaties van hemopoietische cellen waaronder normaal beenmerg, hemopoietische tumorcellen en leukemische cellijnen. De resultaten van studies aan normaal beenmerg toonden aan dat het menselijke IL-3 de proliferatie van multipotente voorlopercellen uit het beenmerg stimuleert en de produktie van myeloide voorlopercellen in het beenmerg activeert. Tevens bleek IL-3 in de meeste gevallen in staat te zijn om de groei van leukemische blastcellen van de mens te stimuleren. Het menselijke IL-3 was in deze studies werkzaam bij een concentratie van ongeveer 5 x 10^{-11} mol per liter. De *in vitro* studies toonden aan dat de biologische activiteiten van het menselijke IL-3 en het IL-3 van de muis met elkaar overeenkwamen. Daaruit werd geconcludeerd dat het menselijke IL-3 zowel in genetische structuur als in biologisch opzicht een grote gelijkenis met het IL-3 van de muis vertoont.

De produktie van grote hoeveelheden goed gedefinieerd menselijk IL-3 (hoofdstuk 3) leidde tot preklinische *in vivo* studies in verscheidene *Macaca* soorten (rhesus en cynomolgus apen). Deze studies waren erop gericht om potentiële therapeutische toepassingen te identificeren. Toediening van het menselijke IL-3 aan dit soort apen resulteerde in een geringe toename van het aantal perifere bloedcellen. Deze geringe activiteit van het menselijke IL-3 in apen zou mogelijkerwijs toegeschreven kunnen worden aan de soortspecificiteit van IL-3. Om deze hypothese te toetsen werd er besloten om het homologe IL-3 van de rhesusaap te isoleren. De klonering van het genomische IL-3 gen van de rhesusaap en de constructie van een IL-3 cDNA van de rhesusaap (RhIL-3) worden beschreven in hoofdstuk 4. De nucleotide sequentie van het RhIL-3 gen vertoont ongeveer 93% homologie met het overeenkomende deel van het menselijke IL-3 gen. Beide genen hebben een identieke genomische organisatie.

Potentiële regulatie-elementen geïdentificeerd in de IL-3 genen van de muis, rat en mens bleken ook aanwezig in het RhIL-3 gen en wijzen op gemeenschappelijke transcriptiefactoren die betrokken zijn bij de regulatie van IL-3. Het RhIL-3 gen codeert voor een 143 aminozuren lang precursor eiwit en bleek negen aminozuren korter te zijn dan het menselijke IL-3. De overeenkomst tussen de eiwitsequenties van het menselijke IL-3 en RhIL-3 is 89.5% voor het signaalpeptide (19 aminozuren) en 80.5% voor het mature IL-3 eiwit (124 aminozuren). RhIL-3 cDNA werd geconstrueerd nadat een genomisch RhIL-3 fragment in COS cellen tot expressies was gebracht. Produktie van goed omschreven RhIL-3 eiwit kwam tot stand door RhIL-3 cDNA in *B. licheniformis* tot expressie te brengen en het door de bacteriën uitgescheiden RhIL-3 op te zuiveren zoals beschreven voor het menselijke IL-3.

De biologische gegevens zoals gepresenteerd in hoofdstuk 5 toonden aan dat RhIL-3 cDNA codeert voor een functioneel eiwit dat hemopoietische cellen van de mens en van de rhesusaap stimuleert. Een directe vergelijking van de biologische activiteit van het menselijke IL-3 met RhIL-3 op beenmerg van de mens en van de rhesusaap liet verschil zien tussen deze IL-3 varianten, in het bijzonder op beenmerg cellen van de rhesusaap. RhIL-3 bleek ongeveer 100 maal actiever te zijn dan het menselijke IL-3 in de stimulatie van hemopoietische voorlopercellen van de rhesusaap, daarentegen vertoonden het menselijke IL-3 en RhIL-3 een vergelijkbare stimulerende activiteit op normale en maligne hemopoietische cellen afkomstig van de mens. Deze vergelijkende studies lieten een uni-directionele soortspecificiteit zien voor het menselijke IL-3 hetgeen dus tevens verklaart waarom er slechts geringe effecten werden waargenomen in de preklinische studies met het menselijke IL-3 in *Macaca* soorten. Behandeling van rhesusapen met RhIL-3 en het menselijke IL-3 toonde onomstotelijk aan dat de preklinische studies met het menselijke IL-3 in apen aanzienlijk beïnvloed werden door de soortspecificiteit van het menselijke IL-3 en daardoor niet representatief zijn voor de potentiële effecten van IL-3 indien toegediend bij de mens.

Om de analyse van de evolutie van IL-3 in primaten mogelijk te maken was het noodzakelijk om de IL-3 coderende sequenties van de chimpansee, marmoset en tamarin te identificeren zoals beschreven in hoofdstuk 6. Het IL-3 van de chimpansee vertoont 98.7% eiwithomologie met het menselijke IL-3. De eiwithomologie tussen IL-3 van de marmoset of tamarin en het menselijke IL-3 bedraagt ongeveer 70%. Evenals RhIL-3 missen IL-3 van marmoset en tamarin de laatste negen C-terminale residuen. IL-3 van mens en chimpansee bleken even actief in het stimuleren van de proliferatie van IL-3 afhankelijke menselijke AML-193 cellen. Daarentegen bleek IL-3 van de tamarin een sterk verminderde (± 900 maal) activiteit te vertonen op deze menselijke cellen. De "multiple alignement" van de coderende IL-3 sequenties van de muis, rat, marmoset, tamarin, rhesusaap, gibbon, chimpansee en mens liet zien dat er slechts enkele kleine gebieden geconserveerd zijn tijdens de evolutie van IL-3.

Analyse van mutanten van het menselijke IL-3, natuurlijk voorkomende varianten van andere diersoorten en een groot aantal geconstrueerde IL-3 chimeren tussen chimpansee en tamarin maakten de identificatie van essentiële domeinen van IL-3 mogelijk (hoofdstuk 6). In deze studies werd de aanwezigheid van minstens twee receptor-bindende domeinen (Fig. 6.4: residuen 47-53 en residuen 132-143) verondersteld.

Phylogenetische stambomen geconstrueerd van afstandmatrixen van coderende IL-3 sequenties vertoonden grote verschillen in de evolutiesnelheid van IL-3 tussen verschillende diersoorten. De snelheid van accumulatie van nucleotide substituties in knaagdieren bleek aanzienlijk hoger dan die in primaten. Verder werd duidelijk dat de accumulatiesnelheid van nucleotide substituties afnam naarmate de "hominoid"-evolutie vorderde. Onderzoek naar de frequentie van nucleotide substituties op synonieme lokaties (mutaties die geen aminozuur verandering teweeg brengen) en niet-synonieme lokaties (mutaties die wel leiden tot een aminozuur verandering) onthulde een ongewone distributie van deze twee typen substituties in de meeste IL-3 soortvarianten. De extremen waren RhIL-3 met slechts enkele synonieme substituties en IL-3 van de gibbon met vrijwel alleen synonieme substituties. In RhIL-3 overtrof het aantal niet-synonieme substituties zelfs het aantal synonieme substituties, hetgeen niet eenvoudig te verklaren valt vanuit een willekeurig optreden van mutaties. Op grond daarvan werd gepostuleerd dat het merendeel van de aminozuur veranderingen die optraden tijdens de evolutie van IL-3 en die tevens een functionele soortspecificiteit tot gevolg had, een selectieve achtergrond heeft die waarschijnlijk zijn grondslag vindt in de structuur van de uit meerdere subeenheden bestaande IL-3 receptor. De belangrijkste drijvende kracht tijdens de evolutie van IL-3 zou mogelijk de β subeenheid van de IL-3 receptor kunnen zijn. Behoud van functie tijdens de evolutie vereist van de β subeenheid (binden van ligand en associëren met de α subeenheid, wat moet resulteren in een juiste signaal transductie) aanpassing aan veranderingen in drie onafhankelijk evoluerende groeifactoren (IL-3, IL-5 en GM-CSF) en aan evolutie-gebonden adaptaties van de α subeenheid welke deel uitmaakt van het IL-3 receptor complex. Het eigenaardige evolutiegedrag van RhIL-3 (de fixatie van een groot aantal niet-synonieme substituties) biedt een verklaring voor de verminderde binding van het menselijke IL-3 aan de IL-3 receptor op cellen van de rhesusaap.

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CURRICULUM VITAE

Hermanus Burger was born in 's-Gravenhage, The Netherlands, on June 13, 1956. He completed his secondary education (HAVO, Groen van Prinsterer College, 's-Gravenhage) in 1974. After studying chemical technology for one year (Gemeentelijke HTS, 's-Gravenhage) he started to work as a laboratory worker in the chemical industry (Premier Inktchemie, 's-Gravenzande). Meanwhile, he was trained as a technician (biochemistry) at the "Van Leeuwenhoek Instituut" in Delft. This training was completed in 1979. In 1979 he was appointed technician at the Radiobiological Institute (RBI) of TNO Health Research in Rijswijk (Director prof. dr D.W. van Bekkum) and from 1980 to 1985 involved in projects in the field of experimental hematology supported by ZWO. Thereafter, he continued his work at the experimental hematology department and joined the senior technical staff of the RBI. Under the supervision of dr G. Wagemaker and dr ir L.C.J. Dorssers he was introduced to the fields of hemopoiesis and molecular biology, respectively, and engaged in the identification and characterization of interleukin-3. The results of the studies on interleukin-3 are presented in this thesis. From 1991 to 1993 he was appointed scientist in the Gene Therapy department (headed by prof. dr D. Valerio) of the Institute of Applied Radiobiology and Immunology (ITRI) of TNO Health Research in Rijswijk and involved in several projects in the field of gene therapy, transgenic mice and multidrug resistance. In 1993 he was appointed molecular biologist at the Medical Oncology department (headed by prof dr G. Stoter) of the Dr. Daniel den Hoed Cancer Center in Rotterdam.

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Herman Burger