Microanalysis of Gene Expression in Cultured Cells

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Das Wohltemperierte Klavier

I^E Präludien und Fugen

Bach

CONTENTS

ABBREVIATIONS

α-glu	α-glucosidase
β-gal	β-galactosidase
β-hex	β-N-acetylhexosaminidase
CAP	chloramphenicol
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
ER/Golgi	endoplasmic reticulum/Golgi complex
%ERY	nuclei of chick erythrocytes in heterokaryons expressed as percentage of total nuclei scored
GAL^-	β-galactosidase deficient fibroblasts of a patient with G_{M1}-gangliosidosis
GLU^-	α-glucosidase deficient fibroblasts of a patient with Pompe's disease
G6Pase	glucose-6-phosphatase
G6PD	glucose-6-phosphate dehydrogenase
HAU	hemagglutination units
% HET.K	nuclei in heterokaryons expressed as percentage of total nuclei scored
$HEX\ A^-$	β-hexosaminidase A deficient fibroblasts of a patient with Tay-Sachs disease
$HEX\ A^-B^-$	β-hexosaminidase A and B deficient fibroblasts of a patient with Sandhoff's disease
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
mRNA	messenger ribonucleic acid
4-MU	4-methylumbelliferone
MW	molecular weight
NORs	nucleolar organizer regions
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
TPA	12-O-tetra decanoylphorbol 13 acetate
UDS	unscheduled deoxyribonucleic acid synthesis
UV	ultraviolet
XP	xeroderma pigmentosum

GENERAL INTRODUCTION

1.1 Scope of the thesis

The study of gene expression in cultured cells is of importance both in basic cell biology and genetics and for the development of diagnostic techniques for inborn errors of metabolism. This thesis deals with both aspects.

The expression of a variety of enzyme activities and of structural proteins in cultured human fibroblasts and amniotic fluid cells offers the possibility of early diagnosis of patients with a genetic defect involving one of these (enzyme) proteins (for reviews, see Stanbury, et al., 1978; Galjaard, 1980). A prenatal diagnosis in a pregnancy at risk for a genetic metabolic disease is based on a comparison of data obtained after biochemical analysis of cultured amniotic fluid cells and fibroblasts from normal, heterozygous and affected individuals or fetuses. A reliable distinction between these categories is possible only if the conditions of cell cultivation, the cell types to be analyzed and the assay procedures are similar.

One of the first aims of the experimental work described in this thesis was to investigate whether the different types of epithelioid and fibroblast-like cells known to be present in amniotic fluid cultures (Uhlendorf, 1970; Hoehn et al., 1974, 1975) express the same or different activities of a number of lysosomal enzymes involved in genetic metabolic diseases. Since various cell types can be present within the same cell culture we have applied microchemical techniques that enabled enzyme assays to be performed on isolated single cultured cells (van der Veer et al. 1978). The use of such microtechniques is also advantageous in the practice of prenatal diagnosis of metabolic disease. Biochemical assays can be performed on relatively few cultured cells and hence the time interval between amniocentesis and the diagnosis can be reduced as was shown by our work on pregnancies at risk for the lysosomal storage disease G_{M1}-gangliosidosis (Kleijer et al., 1976; Galjaard et al., 1977).

At present some eighty inborn errors of metabolism are known to be expressed in cultured fibroblasts and amniotic fluid cells (for review

see Milunsky, 1979; Galjaard, 1980). A total of 201 genetic enzyme deficiencies are, however, known in man (McKusick, 1982) but most of these are expressed in one or few specialized cell types and can thus not be diagnosed by enzyme assays of cultured fibroblasts or amniotic fluid cells. The scope of prenatal diagnosis would of course be widened if the particular gene function could be activated in cultured cells. We have tried to do so for glucose-6-phosphatase which is deficient in glycogenosis type I, a carbohydrate storage disease of autosomal recessive inheritance. Normally, the activity of glucose-6-phosphatase is expressed mainly in hepatocytes and kidney epithelium. We have tried to induce the expression of this enzyme deficiency in cultured (amniotic fluid) cells by somatic cell hybridization (Galjaard et al., 1977) but these attempts have so far been unsuccessful. Other investigators followed a similar approach to allow in utero detection of genetic diseases involving differentiated functions in specific cell types and organs (Darlington et al., 1980).

Despite the impressive advances in molecular biology our knowledge about the regulation of gene expression is still very limited. The second part of the experimental work described in this thesis concerns studies on the induction of gene expression. As a model system we have used fusions of chick erythrocytes and different types of human mutant fibroblasts derived from patients with an inborn error of metabolism. The mature chick erythrocyte expresses very few constitutive and differentiated markers but reactivation of several functions has been found after hybridization with other cell types (for reviews see Ringertz & Savage, 1976; Sidebottom & Deak, 1976). We have studied the reactivation of DNA repair in chick erythrocytes and found suppression of the human DNA repair process in heterokaryons after fusion of chick erythrocyte and normal human fibroblasts (van der Veer & Bootsma, 1982). Fusion of chick erythrocytes with DNA repair deficient human fibroblasts derived from different types of patients with xeroderma pigmentosum revealed interesting patterns of restoration of the deficient function (Bootsma et al., 1982).

Finally we have used the same hybridization system to study the regulation of the cytosolic enzyme glucose-6-phosphate dehydrogenase, and of a number of lysosomal enzymes which may be involved in inborn

errors of carbohydrate or ganglioside metabolism (Van der Veer et al., 1982). These latter studies also provided information about the species specificity of an early step in the post-translational processing of lysosomal enzymes. This is especially interesting since it was recently found that a genetic defect in this early modification step is responsible for the multiple lysosomal enzyme deficiency in 'I-cell' disease, an autosomal recessive disease associated with severe physical and mental handicaps (Hasilik et al., 1981; Reitman et al., 1981).

The following section of this chapter will deal with a general introduction of gene functions in cultured cells, the genetic deficiencies of various types of markers as used in our studies, and of the induction of gene expression using somatic cell hybridization. Also a short review will be given of micro-analysis of single cultured cells since these techniques have been used both in our work on prenatal diagnosis of genetic metabolic disease and in our hybridization studies, where a mixture of different fused and non-fused cell types are present.

1.2 Gene functions in cultured cells

The fundamental laws of heredity were discovered by Gregor Mendel in 1866. He deduced that the hereditary characters of the pea are carried and passed on to the progeny as discrete units. Without knowledge of these experiments Wilhelm Roux postulated a linear arrangement of hereditary units along the chromosomal threads (see Weismann, 1891). The rediscovery in 1900 of Mendel's work could now be understood in terms of behaviour of chromosomes in mitosis and meiosis.

Classical genetics led to the concept of the gene as the fundamental biological unit of heredity and postulated that it must possess the following basic properties. It had to have a specific function in the cells and hence in the organism as a whole. It had to be capable of exact self replication. Although usually an extremely stable entity it had to be susceptible to occasional sudden change or mutation. The discoveries of e.g. Avery in 1944 that genes are embodied in DNA and the elucidation of the molecular structure of this substance by Watson, Crick and Franklin in 1953, made it possible to begin to understand the nature of genetic diversity. The development of molecular genetics

led to the concept of the gene as being represented by a length of DNA, containing several hundred or thousand base pairs, and having its own unique structure from which is derived its functional specificity. A gene mutation can be envisaged as a consequence of some event that results in an alteration of the base pair sequence of a particular gene. The inherited information coded in the genes defines the structure of all the enzymes and other proteins in the cells. The genes determine not only the structure of proteins but they are also involved in the regulation of their synthesis.

Cell culture as an experimental procedure had its inception in the demonstration by Harrison in 1907 that nerve fibres would grow out from fragments of frog spinal cord isolated in clotted lymph (Harrison, 1907). Methods were later developed by Carrell, Warren, Lewis and others for serial propagation of various kinds of cells in vitro (see Lewis, 1927). During the early period of experimentation with cultures much was learned about the characteristics of isolated populations of living cells. For instance, the failure of most serial cultures to resemble their tissue of origin limited the numbers of characteristics that could be studied in the cells. Krooth (1962) used as a 'rule of thumb' that an enzyme is likely to be ubiquitous and therefore present in cultured cells, if that enzyme is present in red or white blood cells and in a parenchymatous organ other than the spleen. Ephrussi (1972) made a distinction between ubiquitous, household functions, i.e. metabolic functions essential for the maintenance and growth of any cell, and differentiated, facultative functions which must be necessary for the survival of the multicellular organism and of the species but not for that of the cells. Another term for ubiquitous functions which has been used is constitutive markers (Ringertz & Savage, 1976). It is, however often difficult to make a clear distinction between constitutive and differentiated markers and it has therefore been suggested that both may be the extremes of a continuum (Ringertz and Savage, 1976). During the last decades cultured normal fibroblasts and tumor derived cell lines have been extensively studied. An estimate for the number of genes active in a given tissue or cell type can be provided by the number of different messenger RNA species. The number of different messengers found in a mammalian cell populat-

ion is, generally, of the order of 10 000 and only a small proportion of these may be specific for the cell type (Lewin, 1980).

1.3 Deficiencies in gene functions

The last catalogue of human inherited abnormalities (McKusick, 1978) lists about three thousand conditions which are thought or proven to be subject to Mendelian inheritance. The responsible molecular defect has been elucidated in less than 10% of these conditions. Many investigations have been made to trace the particular constellation of clinical abnormalities that are observed back to the effects of some specific enzyme or protein defect resulting from a single gene mutation. For the great majority of inherited diseases, however, we have as yet a far from clear understanding of the sequence of events from gene mutation, modified synthesis or structure of a specific protein, secondary metabolic and cell biological consequences and how these changes give rise to clinical signs and symptoms. (McKusick, 1972; Scriver & Rosenberg, 1973; Nyhan, 1974; Grisolia et al., 1975; Cornblath & Schwartz, 1976; Hanawalt et al., 1978; Stanbury et al. 1978; Sperling et al., 1979; Galjaard, 1980; Callahan & Lowden, 1981).

1.3.1 Constitutive Markers

1.3.1.1 Lysosomal enzyme deficiencies

Lysosomal enzymes are kept apart from the rest of the contents of the cell by a single membrane, which effectively prevents leakage of the hydrolases into the surrounding cytoplasm (De Duve et al., 1963; Dingle & Fell, 1969). Characteristically, these lysosomal hydrolytic enzymes have rather low pH optima and each has its own specificity directed at a particular type of linkage. Together they are involved in the degradation of a wide variety of different complex macromolecules, which may be polysaccharide, lipid, protein or nucleic acid in nature. Such macromolecules may originate from the components of the cell itself or may be taken up from the outside by the process of endocytosis. In most instances genetic lysosomal enzyme deficiencies were discovered only after the accumulating macromolecules had been identified. (Stanbury et al., 1978; Galjaard, 1980; Callahan & Lowden, 1981).

An autosomal recessive glycogen storage disease, with profound hypotonia and cardiac failure leading to death during the first years of life was first recognized by Pompe in 1932 (Pompe's disease). In 1957 Cori classified this disease as glycogenosis type II on biochemical criteria and Hers (1963) discovered that the responsible molecular defect was a deficiency in acid α-glucosidase (α-glu). Infantile, juvenile and adult forms of glycogenosis type II have since been reported (reviews: Cornblath & Schwartz, 1976; Howell, 1978; Loonen, 1979; Galjaard, 1980). The molecular basis for this heterogeneity has not yet been resolved, but in fibroblasts from patients with the infantile form of glycogenosis type II the activity of α-glucosidase is 1% of control values or lower, whereas in fibroblasts from the adult form a residual activity of 10-20% is found (Reuser et al., 1978; see also Martin er al., 1976; Mehler & DiMauro, 1977). There is recent evidence of a reduced rate of synthesis of α-glu molecules in fibroblasts from patients with the late-onset form of Pompe's disease (Reuser & Kroos, 1982 submitted for publication). In human fibroblasts α-glu is synthesized as a precursor with a molecular weight (MW) of 95 000 which is processed through a number of intermediates to a variety of shorter chains of which the major components have a MW of 79 000 and 76 000 (Hasilik & Neufeld, 1980).

Okada & O'Brien (1968) were the first to demonstrate that a deficiency of acid β-galactosidase (β-gal) is the underlying genetic defect in G_{M1}-gangliosidosis. β-gal is normally involved in the degradation of G_{M1}-gangliosides and of keratan sulphate-like mucopolysaccharide. In G_{M1}-gangliosidosis accumulation of G_{M1}-gangliosides in the brain is the characteristic feature (O'Brien, 1972). The progressive destruction of neuronal cells due to accumulation of these compounds in the lysosomes accounts for the profound cerebral degeneration. Lysosomal inclusions are also seen in histiocytes and parenchymal cells of liver and spleen, which can be due to storage of a keratan sulphate-like mucopolysaccharide, but also the G_{M1}-gangliosides content is increased in these cells (Suzuki et al., 1971). G_{M1}-gangliosidosis is a relatively rare autosomal recessive disorder and during the last decade several clinical forms (infantile, juvenile and adult) have been reported (for reviews: Suzuki et al., 1977; O'Brien, 1978;

Lowden & O'Brien, 1979).

In extracts of human liver β-gal is found as a monomer with a MW of 70 000 and, in addition, aggregates with a MW of 600 000 - 800 000 are encountered (Norden et al., 1974; Hoeksema et al., 1979). Recently Skudlarek & Swank (1979) have demonstrated that in the mouse macrophages β-gal is synthesized in the form of a 82 000 MW precursor which is converted to a form with a MW of 63 000. Similar results were obtained by D'Azzo et al. (1982 in press) for human fibroblasts where a 85 000 MW precursor was found to be processed to a 64 000 MW mature β-galactosidase. In normal fibroblasts the half-life of β-galactosidase is approximately 10 days and the same time for the residual activity in G_{M1}-gangliosidosis cells (Van Diggelen et al. 1981, 1982). The enzyme polypeptide is coded by a gene on chromosome 3 and the infantile, juvenile and adult types of G_{M1}-gangliosidosis are due to allelic mutations in this structural gene. There is contradiction about the role of chromosome 22 in the expression of human β-gal (Bruns et al., 1977; Shows et al., 1977; de Wit et al., 1979; Hoeksema et al., 1980).

Tay-Sachs disease and Sandhoff disease are clinically and pathologically very similar disorders, characterized by the accumulation of G_{M2}-ganglioside (reviews: Adachi et al., 1978; O'Brien, 1978; Sandhoff & Christomanou, 1979; Galjaard, 1980). The deficient enzyme is β-hexosaminidase (N-acetyl-hexosaminidase E.C. 3.2.1.30) which normally hydrolyses β-N-acetyl glucosaminyl - as well as β-N-acetyl galactosaminyl linkages. Two major isozymes of β-hexosamidase (β-hex) known as β-hex A and β-hex B have been identified (Robinson & Stirling, 1968) and a minor acid β-hex S isozyme is found (Ikonne & Desnick, 1974). They can be separated by ion-exchange chromatography, isoelectric focusing or electrophoresis. β-Hex A is a more acidic and thermolabile form than β-hex B in tissues and cells from control persons. The mature isozyme B is a tetrameric enzyme composed of two dimers. These dimers are generated from a single precursor β-chain that is subsequently cleaved proteolytically into two fragments with a MW of ± 29 000 that remain attached by disulphide links (Hasilik & Neufeld, 1980; Mahuran & Lowden, 1980). The minor isozyme S is thought to contain only α-chains. Mahuran and Lowden (1980) suggested that Hex A is an oligomer consisting of one α-chain and two smaller β-chains. Pulse-

labelling of human fibroblasts with (^{3}H) leucine followed by immunoprecipitation with antisera raised separately against the α- and β-subunits of placental β-hexosaminidase A allowed the identification of a 67 000 chain as a precursor form of the mature 54 000 α-chain. A 63 000 β-precursor form is processed via 61 000 and 52 000 intermediate forms into 29 000 β-chains (Hasilik & Neufeld 1980). Labelling patterns of fibroblasts derived from patients with Tay-Sachs disease, which are deficient in β-hex A or Sandhoff disease, deficient in both Hex A and Hex B as result of a deficiency in the β-subunit, confirm the molecular structure of the hexosaminidases described above.

I-cell disease was first described by Leroy & De Mars (1967) who demonstrated an abundance of peculair cytoplasmic granular inclusions in cultured fibroblasts of the patient; this resulted in the designation of the fibroblasts as inclusion cells or 'I-cells'. The pathological symptoms of I-cell patients are early cessation of increase in stature, gingival hyperplasia, psychomotor retardation and coarsening of facial features. Death occurs mostly in the first decade of life and is usually attributed to pneumonia or congestive heart failure (Martin et al., 1975; Farriaux et al., 1976; Miller et al., 1981). A remarkable feature of this disease was the intracellular deficiency of more than ten lysosomal hydrolases and the increased activity of the same enzymes in serum and in the medium in which mutant fibroblasts were cultured (Wiesmann et al., 1971; Leroy et al. 1972).

Recently Hasilik et al. (1981) and Reitman et al. (1981) discovered that the primary defect in I-cell disease is a genetic deficiency of N-acetyl glucosamine-1-phosphotransferase. This enzyme, which is located in the ER/Golgi region phosphorylates mannose residues on precursor forms of lysosomal glycoproteins. The phosphotransferase deficiency interferes with correct compartmentalization and the lysosomal enzymes are excreted. These extracellular enzymes were already known to be "recognition defective" hydrolases (Hickman and Neufeld, 1972).

The study of these mutant human cells have contributed much to our insight in the complexity of the post-translational modification of lysosomal glycoproteins. At present the following steps for the transport of newly synthesized acid hydrolases to the lysosomes is suggested (Blobel, 1980; Neufeld, 1981; Sly et al., 1981; d'Azzo, 1982). Pre-

cursor polypeptides are made on membrane bound ribosomes and a hydrophobic leader sequence expedites the transfer of the molecules to the intracisternal space of the endoplasmic reticulum, where it is subsequently removed (Erickson & Blobel, 1980; Sabatini et al., 1982). In the cisternal space the precursor molecules are glycosylated and phosphorylated enzymatically by transfer of N-acetyl glucosamine-1-phosphate from UDP-N-acetyl glucosamine to mannose-containing oligosaccharide side chains (Hasilik et al., 1981 and Reitman et al., 1981). A phosphodiesterase cleaves the phosphodiester bound releasing N-acetyl glucosamine and exposing the mannose-6-phosphate recognition marker (Varki & Kornfeld, 1980) for which there is a specific membrane receptor (Hickman & Neufeld, 1972; Kaplan et al., 1977). This receptor/mannose-6-phosphate system is required for a correct intracellular compartmentalization so that the precursors of the lysosomal enzymes from the ER/Golgi region are transferred into the lysosomes. The membranes with bound precursors give rise to vesicles that eventually become the primary lysosomes (Neufeld, 1981; Sly & Creek, 1981).

It is not known exactly where the assembly of oligomeric enzymes and the maturation of the precursor molecules take place, but several studies suggest that the final maturation occurs inside the lysosomes (Halley, 1980; Frisch & Neufeld, 1981; Neufeld, 1981; Tager et al., 1981; Sabatini et al., 1982; Sly & Fisher, 1982).

1.3.1.2 Glucose-6-phosphate dehydrogenase, a cytosolic enzyme deficiency

The constitutive marker glucose-6-phosphate dehydrogenase (G6PD) is a cytoplasmic enzyme involved in the direct oxidative pathway of carbohydrate metabolism. G6PD catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconate and this is accompanied by the concommitant reduction of the coenzyme $NADP^+$ to NADPH, which acts as electron donor in reductive biosyntheses. The discovery of inherited variation of G6PD followed the finding that a significant proportion of American Negroes developed acute hemolysis after receiving the synthetic antimalarial drug primaquine (Hockwald et al., 1952). The hemolytic response was due to an intrinsic red cell abnormality (Dern et al., 1954) as a result of a specific deficiency of G6PD

(Carson et al., 1956). Several other drugs were found to induce hemolytic crises in G6PD deficient persons (see: WHO Technical Report Series no. 366, 1967). This deficiency is also the underlying cause in the nutritional sensitivity known as favism (Larizza et al., 1958; Zinkham et al., 1958). More than 140 different variant forms of G6PD enzyme have now been discovered (Yoshida & Beutler, 1978). They differ from the normal form of the enzyme in electrophoretic mobility, Michaelis constants, thermostability, and pH optimum. It seems probable that most or all of them are due to single amino acid substitutions (Yoshida 1967; Yoshida & Beutler, 1978). The variants are apparently determined by a series of alleles at a gene locus on the X-chromosome (Childs et al., 1958, Human Gene Mapping Conference V, 1979).

1.3.1.2 Xeroderma pigmentosum. An inherited disorder in nucleic acid metabolism.

DNA repair processes are essential for the maintenance of inherited information, because the DNA in living cells is continually subject to alterations, either spontaneous or induced by chemicals or radiation. One of these repair processes is dimer excision repair (see Figure 1), which is genetically deficient in xeroderma pigmentosum (XP) (for reviews see: Arlet & Lehman, 1978; Bootsma, 1978; Friedberg et al., 1979; Hanawalt et al., 1979; Pawsey et al., 1979; Kraemer, 1980; Lehman & Karran, 1981).

XP is a rare autosomal-recessive skin disease with hypersensitivity to ultraviolet radiation and was first described by Hebra & Kaposi (1874). Initially, it was thought that the defect was a structural gene mutation for a putative repair endonuclease , because XP cells are not sensitive to agents that produce strand breaks. However, when unscheduled DNA synthesis (UDS) was examined in the nuclei of heterokaryons after fusion of two different XP fibroblast strains, certain combinations showed a normal excision repair capacity (de Weerd-Kastelein et al., 1972). Seven complementation groups have so far been defined on this basis, designated A-G (Bootsma, 1978). There is considerable evidence that in all seven groups there is some defect in the incision step. Support comes from studies by Fornace et al.

(1976), who studied directly the frequency of strand breaks following UV irradiation using the DNA alkaline elution technique and from some studies on sedimentation velocity of DNA in alkaline sucrose gradients (Cook et al., 1978; Erixon & Ahnstrom, 1979).

Further evidence comes from the studies of Tanaka et al., 1975, 1977) with T_4 endonuclease V . They permeabilized XP groups A - E cells with UV inactivated Sendai virus and exposed the cells to a dimer specific incision enzyme from bacteriophage T_4. After this treatment restoration to normal levels of UDS was shown. In as yet unpublished experiments in which T_4 endonuclease V or Micrococcus luteus UV endonuclease was introduced by microinjection, restoration of UDS could also be demonstrated in XP groups F and G (A.J.R. de Jonge, personal communication).

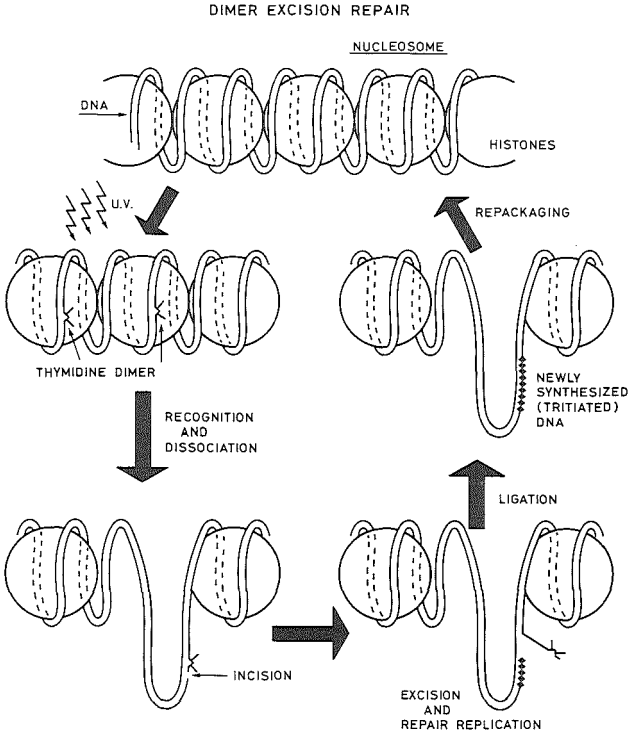

Fig.1 Schematic representation of the steps involved in excision repair of damaged DNA by ultraviolet light. DNA, newly synthesized in the presence of (^{3}H) thymidine can be recognized by autoradiography.

Studies on the kinetics of complementation revealed that cells from group XP-A are complemented rapidly even in the absence of protein synthesis. Cells from group XP-C and XP-D are complemented more slowly and probably 'de novo' synthesis of the correcting normal gene product is required (Pawsey et al., 1979; Matsukuma et al., 1981). Differences in kinetics of complementation are also found in cybrid studies (Keijzer et al., 1982 in press). Mortelmans et al. (1976) postulated that the XP-A mutation is in a gene, the product of which modifies the chromatin structure to facilitate the accessibility for repair enzymes. They presented evidence that normal human cells disrupted by sonication were able to excise pyrimidine dimers both from naked exogenous DNA and from DNA in the cell's own chromatin. Extracts of XP group A cells were also able to excise dimers from naked DNA, but not from DNA in its own chromatin. Confirmation of the results of Mortelmans have so far been unsuccessful.

1.3.2 *Glucose-6-phosphatase, a differentiated marker and its deficiency in glycogenosis type I*

The glycogen storage disease, first described clinically by Von Gierke (1929) was later found to be due to a glucose-6-phosphatase (G6Pase) deficiency (Cori and Cori, 1952). Glycogenesis type I (Von Gierke disease) is an autosomal recessive disorder, with storage of glycogen in liver and kidney; acidosis, growth retardation and recurrent episodes of hypoglycemia. G6Pase is a multifunctional enzyme (for review see Nordlie, 1979) showing the ability to hydrolyze as well as synthesize glucose-6-phosphate. The physiological role of this enzyme is to catalyze the hydrolysis of glucose-6-phosphate as the terminal step in both gluconeogenesis and glycogenolysis in liver and kidney (Fanth & Rome, 1945; de Duve et al., 1949; Swanson, 1950). G6Pase catalyzes transfer of phosphate from inorganic pyrophosphate to glucose, thereby forming glucose-6-phosphate, and has the ability to cleave inorganic pyrophosphate hydrolytically (Stetten & Taft, 1964; Nordlie, 1974). This endoplasmic reticulum bound enzyme is expressed in the liver and kidney, although minor activities have also been found in intestine, pancreas and the adrenals (for review see Nordlie, 1979).

1.4 Induction of Gene Expression

1.4.1 Introduction

A variety of techniques are available for the study of the induction of gene expression in eukaryotic cells. The method of somatic cell hybridization has been used most often to investigate the induction or suppression of constitutive or differentiated markers (Harris, 1970; Ephrussi, 1972; Davidson, 1974; Davidson & de la Cruz, 1974; Bernard, 1976; Ringertz & Savage, 1976). Other approaches have been microcell-mediated gene transfer (Ringertz & Savage, 1976; Fournier & Ruddle, 1977) and chromosome-mediated gene transfer (McBride & Ozer, 1973; Klobutcher & Ruddle, 1981). DNA-mediated gene transfer using the calcium-phosphate precipitation method (Graham & v.d. Eb, 1973; Bacchetti & Graham, 1977; Maitland & Mc Dougall, 1977; Wigler et al., 1977; Willecke et al., 1979) and micro-injection (Graessmann, 1968, Diacumakos et al., 1970, Capecchi, 1980) has recently received an important stimulus by the development of recombinant-DNA techniques (Old & Primrose, 1981; Williamson, 1981[a,b]).

A difference between these methods and somatic cell hybridization is the amount of donor genomic material transferred. A whole genome is transferred by cell hybridization, a few intact chromosomes by microcells, subchromosomal segments by chromosome-mediated gene transfer and DNA fragments in the kilobase range in DNA-mediated gene transfer. Single genes can now be transferred using recombinant DNA methodology. Since we have used somatic cell hybridization in the experimental work described in this thesis we will limit the further introduction to this subject.

1.4.2. Heterokaryons

Two models are discussed in which induction of gene expression is the central theme:

- -the reactivation system, which concentrates mainly on constitutive markers
- -a model for induction of a differentiated marker

Heterokaryons are multinuclear cells with nuclei from two different cell types. Heterokaryons exhibiting a marker of one of the parental

cells are said to show expression; failure to express the marker is referred to as extinction. The term coexpression is used when there is simultaneous expression of homologous genes of both parental genomes. In early cell fusion experiments Harris (1965) found that nuclei in differentiated cells such as macrophages, lymphocytes and avian erythrocytes could be induced to resume or increase the synthesis of RNA and DNA, the activities of which had been 'turned off' during differentiation. This is referred to as reactivation.

DNA synthesis and ribosomal RNA synthesis are not detectable in mature chick erythrocytes at their final stage of differentiation (Cameron & Prescott, 1969; Ringertz & Bolund, 1974; Zentgraf, 1975). At the same time no nucleolar structure can be identified although a small number of resting structures called micronucleoli have been described (Smetana & Likovsky, 1971; Small & Davies, 1972; Smetana et al., 1975; Walmsley & Davies, 1975; Laval et al., 1981). However, it has been reported that mature avian erythrocytes contain small amounts of RNA polymerase II (Kruger & Seifart, 1977; Longacre & Rutter, 1977) and a low level of heterogeneous RNA synthesis has also been detected (Maclean & Madgwick, 1973; Scheintaub & Fiel, 1973; Zentgraf, 1975; Kruger & Seifart, 1977; Tobin et al., 1978).

During the last fifteen years the reactivation of a considerable number of markers has been studied in heterokaryons after fusion of chick erythrocytes and mammalian cells (for reviews see Ringertz & Savage, 1976; Sidebottom & Deak, 1976).

At an early stage after fusion the DNA protein complex undergoes drastic physicochemical changes (Bolund et al., 1969; Ringertz, 1969). Dispersion of the highly condensed chick chromatin and increase of nuclear volume and dry mass occurs (Harris, 1967; Lindberg, 1974; Dupuy-Coin et al., 1976). This increase appeared to be due to an accumulation of human nucleospecific macromolecules (Ege et al., 1971; Ringertz et al., 1971; Appels et al., 1974[a,b]; Goto & Ringertz, 1974). The erythrocyte nuclei preferentially took up mammalian non-histone proteins relative to mammalian histones, but at least one mammalian histone (H1) entered the chick nuclei. At the same time the H5 fraction, specific for avian erythrocytes was lost (Appels, 1974[b]).

Inside the chick nuclei heterogenous nuclear RNA synthesis was found within the first hours after fusion, and the rate increased in a direct relation to nuclear growth. At this early stage RNA was not transported to the cytoplasm (Harris, 1965; Harris & Cook, 1969). There is an increase in nucleoplasmic RNA polymerase activity (Carlsson et al., 1973) but ribosomal RNA (rRNA) could not be detected.

At about 15 hours after fusion ^{3}H-thymidine incorporation was seen in the reactivating chick nuclei (Harris, 1965). Several chick enzymes involved in nucleic acid metabolism were detectable within the first 24 hours after fusion, such as hypoxanthine guanine phosphoribosyltransferase (Bols et al., 1979), adenosine phosphoribosyltransferase (Clements et al., 1975) and cytoplasmic thymidine kinase (Kit et al., 1974). Microspectrophotometric measurements of Feulgen stained cells showed that at 48 hours after fusion many chick nucleoli contained a premitotic amount of DNA (Bolund et al., 1969). However, Johnson & Mullinger (1975) showed that at mitosis many of the erythrocyte nuclei underwent premature chromosome condensation and were destroyed. In some of these cases chick genes (Schwartz et al., 1971; Boyd & Harris, 1973; Klinger & Shin, 1974) or whole chick chromosomes (Kao, 1973) were retained in the daughter cells. Appels et al. (1975) demonstrated that some of the chick erythrocyte nuclei in the heterokaryons remained intact during mitosis and were segregated into one of the daughter cells.

The appearance in electron microscopical studies of prenucleoli as soon as 24 hours after fusion (Dupuy-Coin et al., 1976; Hernandez-Verdun & Bouteille, 1979) is consistent with the immunofluorescence data using nucleolus specific antibodies (Ringertz et al., 1971). More developed nucleoli are detected 2 days after fusion, as first reported by Harris et al. (1969) in light microscopical studies. At 5 days after fusion practically all erythrocyte nuclei in heterokaryons were seen to have developed nucleoli, but interchromatin and perichromatin granules were seldom observed and the nucleoli remained fibrillar (Dupuy-Coin et al., 1976).

The development of nucleoli coincided with the appearance of precursor rRNA and an increase of nucleolar RNA polymerase was detected (Harris et al., 1969; Carlsson et al., 1973). Bramwell (1978) demon-

strated that a significant amount of chick rRNA appears in the cytoplasm of the heterokaryons, but much less than expected from autoradiographic studies.

After fusion of chick erythrocyte with rat L6 myoblasts a new polarization of the cytoplasmic organelles and the development of a Golgi apparatus in the vicinity of the chick nucleus is seen in the heterokaryons (Dupuy-Coin et al., 1976).

With the reappearance of the chick nucleolus in heterokaryons production of chick nucleolar antigens (Ringertz et al., 1971) chick surface antigens (Harris et al., 1969) and chick surface receptors (Dendy & Harris, 1973) begins. In 1969 Harris proposed that the nucleolus is involved in cytoplasmic expression of specific proteins. More recent experiments using in situ hybridization with cDNA confirm this role of the nucleolus (John et al.,1977).

Finally, some heterokaryons give rise to mononucleated synkaryons, and subsequently the proliferating hybrid cells rapidly eliminate most of the chick chromosomes (Schwartz et al., 1971; Boyd & Harris, 1973; Kao, 1973; Trisler & Coon, 1973; Kit et al., 1974; Leung et al. 1975).

So far all markers discussed involve induction of constitutive functions. Early studies did not show induction of differentiated markers. Neither expression of chick myosin was found in the fusion of chick erythrocyte with rat myotube (Carlsson et al., 1973) nor chick serum albumin in the fusion with rat hepatoma (Szpirer, 1974). In mouse A9-chick erythrocyte heterokaryons hemoglobin synthesis was not initiated as judgdd by ^{59}Fe incorporation studies (Harris, 1970). The first successful report on induction of a differentiated marker in reactivated chick nuclei was recently published by Linder et al. (1981). Immunoprecipitation studies using antisera against chicken hemoglobin on ^{35}S-methionine labelled heterokaryons led to the identification of adult chicken α^A, α^B and ß-globin polypeptides.

To enable the detection of a mutation involving a differentiated marker that normally is not expressed in cultured (amniotic fluid) cells Galjaard et al. (1977) suggested a procedure that is illustrated in Fig. 2. The idea was to fuse cultured cells lacking the differentiated marker with mutant specialized cells in the hope that the

latter would provide the necessary regulation to induce expression in the heterokaryon. The procedure had as its aim the prenatal detection of glycogenosis type I where G6Pase, an enzyme that is not normally expressed in cultured amniotic fluid cells, is deficient. The assumption was that fusion of normal amniotic fluid cells with G6Pase deficient hepatocytes could lead to induction of G6Pase activity.
It was assumed that if the fetus were affected by Glycogenosis type I G6Pase activity would not appear. Two prerequisites in this model are that the mutation in Glycogenosis type I is a structural one and that G6Pase activity is not extinguished after fusion of control hepatocytes with amniotic fluid cells.

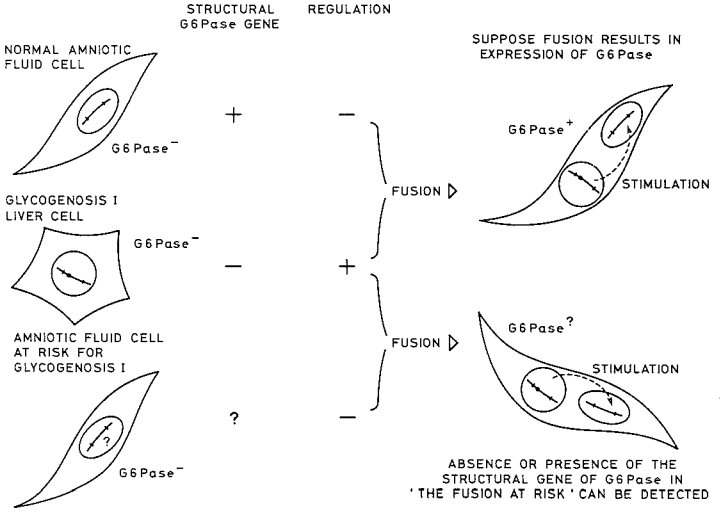

Fig.2 Scheme of cell hybridization experiments designed to induce gene expression in cultured amniotic fluid cells.

There are some disadvantages in using heterokaryons to induce gene expression. For instance they have a limited life-span, which may be too short for the induction of certain genes. Prolongation of the life span of heterokaryons can be extended up to 3 weeks by using chemicals such as mitomycin C (Linder et al., 1981) or röntgen radiation (Sidebottom & Deak, 1976) as inhibitors of DNA replication. There are also technical problems in analyzing heterokaryons in the

presence of homokaryons and unfused cells. Relatively few enzymes can be assayed at the single cell level. A further limitation may be that the interacting genomes are separated by membrane barriers. This is not the case when synkaryons are studied. An advantage of the use of heterokaryons is that the complete genomes of both parental cells are retained and that the selection pressure which is necessary to isolate synkaryons is not required and thus will have no influence on the phenotypic expression. Furthermore, the role of gene dosage can easily be studied in heterokaryons containing varying numbers of parental nuclei.

1.4.3 Synkaryons

Many experiments have been done to investigate gene expression in somatic cell hybrid synkaryons. Most of these studies have involved the expression of differentiated functions. One of the main questions has been and still is whether mammalian cells use diffusible regulator substances to control gene expression (Davidson & Yamamoto, 1968) and if so, how these regulator substances act. The approach in most of the experiments has been to hybridize cells that express a differentiated function with cells that do not express that function, followed by the analysis of the phenotype of the hybrid cell. Such studies have been performed both on intraspecies hybrids and on interspecies hybrids. The latter have the disadvantage of an extensive loss of chromosomes of one of the parental cells. The different functions that have been analyzed include liver- brain - muscle- and kidney-specific functions, pigment synthesis and immunological functions. In most cases the parental cells with differentiated functions were not derived from normal organs or tissues but from a differentiated tumor which in culture often shows an abnormal karyotype. The results obtained in all these studies have been reviewed by Bernhard, (1976) and Ringertz & Savage, (1976). In this thesis the discussion about differentiated markers will focus on possible mechanisms which either induce or extinguish gene expression. The discussion about the constitutive markers will be restricted to rRNA, nucleolar organizer regions (NORs) and histone expression.

Davidson (1978) suggested that the occurrence of extinction or ac-

tivation of differentiated functions in hybrid cells can be interpreted in terms of operation of control mechanisms involving diffusible regulatory substances. Studies of gene dosage effects provided evidence that the expression of differentiated functions in hybrids can be altered by changing the parental genome ratios (Benda & Davidson, 1971). If these phenotypic changes are indicative of the operation of control mechanisms which utilize diffusible regulatory substances, the results of the gene dosage experiments (see Ringertz & Savage, 1976; Davidson, 1978) suggest that such regulatory substances are produced in limited quantities. Only a twofold change in genome ratio in hybrid cells is sufficient to alter the expression of differentiated functions.

The reappearance of extinguished markers as a consequence of chromosome elimination (in interspecies hybrids) has been observed in several cases involving a variety of different cell types and phenotypic markers. The data support the notion that the suppression of gene expression requires the continuous presence of genes that produce diffusible regulator substances.

In situations where extinction of a differentiated function in interspecies hybrids is permanent, the extinction of a structural gene can of course be the result of the loss of that particular chromosome carrying the structural gene coding for the particular marker. Even the most careful karyotype-analysis can not completely rule out this cause of extinction, because of the frequent chromosome rearrangements in vitro and because of the limited resolution of cytological techniques for the indentification of chromosomes. Another difficulty is that an enzyme, although transcribed and translated in the hybrid cells might escape detection as a result of rapid degradation, inhibition or inactivation (Paigen, 1979).

In somatic cell hybrid synkaryons of interspecies origin an extensive chromosome elimination occurs (Weiss & Green, 1967). This chromosome segregation involves preferential elimination of chromosomes of one species, depending not only on the species, but also on the type of parental cell used (Ringert & Savage, 1976). Studies on mouse-human synkaryons show that if human chromosomes are lost, the human nucleolus organizer regions (NORs) and 28S rRNA are extinguished (Elicieri &

Green, 1969; Miller et al., 1976). If mouse chromosomes are lost, mouse NORs and 28 S rRNA are extinguished (Croce et al., 1977). Marshall et al. (1975) demonstrated that heterokaryons between human and mouse and human and hamster cells produce both parental forms of 28S rRNA. Whereas synkaryons produce only the RNA species of those cells whose whole set of chromosomes are retained (see also Lipsick et al. 1981). The loss of cytoplasmic 28S rRNA is correlated with the loss of the production of 45 S precursor rRNA indicating regulation at the level of transcription (Perry et al., 1979).

In mouse-human hybrids that were destined eventually to lose human chromosomes active NORs as indicated by silver staining, were present on both human and mouse chromosomes in metaphase cells harvested during the first few days after fusion. The amount of silver stain on the human NORs decreased gradually and disappeared by about 12 days, presumably reflecting suppression of the transcriptional activity in the human rRNA genes. The time of disappearance depended on the ratio mouse to human chromosomes and on the type of parental cells. It was not closely correlated with loss of human chromosome (Dev et al., 1979).

Soprano et al. (1979) reactivated the 'silent' rRNA genes of either mouse or human origin by infecting the appropriate hybrid cells with SV40. The NORs of the silent parent also reappeared. It is known that SV40 virus codes for diffusible proteins and alters the pattern of cellular protein synthesis (Tegtmeier, 1974). Soprano & Baserga, (1980) demonstrated the same reactivation after treatment of the hybrid cells with 12-O-tetra decanoylphorbol 13-acetate (TPA) and speculate that TPA and the SV40 large tumor antigen may act to stimulate rRNA synthesis in a similar although not necessarily identical fashion. This supports the idea of interchromosome communication by diffusible regulatory factors whose function is to modify chromosomal proteins.

Hohmann (1981) reports a striking resemblance between the regulation of histone gene expression and the production of NORs and 28 S rRNA. In mouse-human hybrids where mouse chromosomes were retained, mouse histones were made and the human histones could remain associated with the human DNA in a conservative manner during replication and become diluted out during progressive rounds of chromosome replication

(Hohmann et al., 1977; Ajiro et al., 1978; Hohmann, 1980; Manser et al., 1980). Studies on the coordination of DNA and histone synthesis strongly suggest that the newly synthesized histones are associated with only one of the two DNA strands made by semi-conservative replication (Riley & Weintraub, 1979). According to Hohmann (1981) 'an interesting way to view this connection between nucleolar function and histone gene expression is to presume that the ultimate processing of histone mRNA, its association with ribosomes and transport to the cytoplasm involves the nucleolus'. It is interesting to note that Harris in 1969, already proposed an important role of the nucleolus in mRNA transport to the cytoplasm.

1.4.4 Cybrids

Somatic cell hybridization techniques combined with enucleation procedures offer the possibility to study the separate role of cell nucleus and cytoplasmic factors involved in the regulation of gene expression. In cybrids, which are the result of fusion of enucleated cells (cytoplasts) with whole cells, the expression of the cytoplast markers generally was found to decrease with time after fusion. (Poste & Reeve, 1971; 1972). Recently Keijzer et al. (1982) have shown that cytoplasts from either normal human fibroblasts or complementing XP fibroblasts are able to correct the DNA repair defect in other mutant cells during a period of about 20 hours. There is an example of retention of certain components of the cytoplasts in cybrids during a long period of several months (Bunn et al., 1974). After fusion of chloramphenicol (CAP) resistant cytoplasts with mouse L cells the CAP resistance which is a mitochondrial gene marker was maintained for a long period in the presence of selective medium.

In the fusion of cytoplasts with chick erythrocytes nuclear swelling and ^{3}H-uridine incorporation in the chick nuclei is detected at 12 hours after fusion (Ladda & Estensen, 1970; Ege et al., 1975). Even the development of nucleoli is seen, but the size and level of organization remains in a premature stage (Dupuy-Coin et al., 1976). At 48 hours after fusion RNA synthesis is negligible.

Another example of cybrid work related to the subject of this thesis is the fusion of cytoplasts derived from human fibroblasts

with a single lysosomal enzyme deficiency with whole cells from patients with multiple lysosomal enzyme deficiencies such as 'I-cell' disease or combined β-galactosidase/neuraminidase deficiency. In all these studies the processing defect underlying the multiple lysosomal enzyme deficiency was partly corrected by the cytoplasts during a period of at least 2-3 days (de Wit-Verbeek et al., 1978; d'Azzo et al., 1980; Jongkind et al., 1981). This implies that the processing factors involved are stable in enucleated cytoplasm during this period.

1.5 Microanalysis in cultured cells

As mentioned earlier the study of gene expression in heterokaryons in the presence of homokaryons and unfused cells may lead to technical problems if the marker cannot be assayed at the single cell level. Early after fusion, if the ratio of heterokaryons to other cells is high, a conventional biochemical assay on the total cell homogenate may give reliable results. It is, however, impossible to quantitate the amount or activity of a particular marker in heterokaryons or synkaryons. Also, if the amount or activity of a marker is low, or if the ratio of unfused cells to heterokaryons or synkaryons is high assays of single hybrid cells are required. Since single cell assays have been used in our experimental work both on prenatal diagnosis of metabolic disease and on gene expression after somatic cell hybridization a short review is given.

Methods for the microchemical assay of microscopically defined structures isolated from freeze-dried cryostat sections were developed by Lowry and others (Lowry, 1953; Matschinsky, 1971; Lowry & Passoneau, 1972). At the same time Glick used a microspectrophotometer for extinction measurements in microdroplets (for reviews see Glick, 1963; 1971; 1977). Also fluorogenic substrates became available for activity measurements of a variety of hydrolytic enzymes (Robinson, 1964; Furth & Robinson, 1965). A combination of these principles and their adaption to cultured cells enabled Galjaard and coworkers to assay the activities of several lysosomal enzymes in small numbers of cultured cells and in some instances in single (hybrid) cells (Galjaard et al., 1974[a,b]; for reviews see Galjaard et al., 1977; Galjaard, 1980). After cultivation in special dishes single or small numbers of freeze-dried

cells can be isolated by free hand dissection under an inverted microscope. The microscopic structure is maintained sufficiently to allow recognition of different cell types within one culture. It is also possible to recognize within one hybrid cell different numbers and types of nuclei. In case different parental cells cannot be distinguished morphologically, the parental cells can be labelled with small or large beads (Ringertz & Savage, 1976).

The microchemical assay can be performed by incubating isolated cells in (sub)microliter volumes of substrate under paraffin oil to prevent evaporation. Depending on the type of assay the extinction or fluorescence can be measured in glass capillaries or directly on microdroplets using a microscope spectrophotometer of microfluorometer (Lowry and Passonneau, 1972; Galjaard et al. 1974[a]; Jongkind et al. 1974; Wudl and Paigen, 1974; for review see Galjaard, 1980).

Microchemical assays on small numbers of cultured human cells have been successfully applied in the prenatal diagnosis of lysosomal storage diseases (Galjaard et al., 1973; 1974[c]; Niermeijer et al., 1975; Kleijer et al., 1976; for reviews see Galjaard et al., 1977 and Galjaard, 1980). It has also been possible to perform lysosomal enzyme assays on single human fibroblasts in co-cultivation and somatic cell hybridization experiments which has made possible the study of transfer of enzymes from normal to mutant human fibroblasts (Reuser et al., 1976[a]) and quantitative complementation analysis on different genetic variants of glycogenosis type II (Galjaard et al., 1975; Reuser et al. 1976[b]).

For those metabolic parameters that can be specifically demonstrated by incorporation of radioactive labelled precursors autoradiography of cultured cells also provides the opportunity of single (hybrid) cell analysis. This approach has, for instance, been used in studies on metabolic cooperation in hypoxanthine-guanine phosphoribosyl transferase deficient fibroblasts derived from patients with Lesch-Nyhan syndrome (for reviews see De Bruyn, 1976; Kelley & Wijngaarden, 1978) and in complementation studies after fusion of different variants of DNA repair defective fibroblasts from patients with xeroderma pigmentosum (de Weerd-Kastelein et al. 1972; for review see Bootsma, 1978). Autoradiography after (^{3}H) hypoxanthine or

(^{3}H) thymidine incorporation following ultraviolet radiation has also allowed rapid prenatal diagnosis of Lesch-Nyhan syndrome (Fujimoto et al. 1968; de Mars et al. 1969; Halley and Heukels-Dully, 1977) and xeroderma pigmentosum (Ramsey et al. 1974; Halley et al. 1979).

EXPERIMENTAL WORK AND DISCUSSION

The study of gene expression in cultured cells in this thesis deals with two aspects:

- the development of diagnostic techniques for inborn errors of metabolism based on a comparison of data obtained after biochemical analysis of cultured amniotic fluid cells and fibroblasts from normal and affected individuals or fetuses
- the induction of gene expression in cultured cells, using somatic cell hybridization techniques.

At present only 80 of the 201 known genetic enzyme deficiencies can be diagnosed by analysis of cultured fibroblasts or amniotic fluid cells (Stanbury et al., 1978; Milunsky, 1979; Galjaard, 1980; McKusick, 1978, 1982). An approach to detect markers which are normally not expressed in cultured cells is the direct analysis of DNA using probes obtained by recombinant-DNA techniques (for reviews see Old & Primrose, 1981; Williamson, 1981[a,b]).
In this thesis another important approach on somatic cell hybridization is used to induce gene expression in cultured cells with the aim to obtain more insight in the regulation mechanisms. If activation of specific markers were be possible it would significantly widen the scope of (prenatal) diagnosis of genetic disease.

2.1 Deficiencies in constitutive gene functions

2.1.1 Amniotic fluid cells

Since the first prenatal diagnosis of genetic metabolic disorders by Nadler (1968) and Fujimoto et al. (1968) the scope has considerably enlarged and the methodology of analysis of amniotic fluid and cultures of amniotic fluid cells has improved (for review see Galjaard, 1980). In amniotic fluid cultures several types of cells have been defined on the basis of their morphology (Uhlendorf, 1970; Sutherland et al., 1974; Hoehn et al., 1974, 1975). Besides the most common types of epithelioid and fibroblast-like cells, different types of large, often multinuclear cells have been described. In poorly growing cultures high numbers of such large cells are usually found, which led

Hoehn et al., (1974) to make the suggestion that these cells are senescent. Since a prenatal diagnosis of a metabolic disease is based on a comparison of analyses of different cultures it is important to know whether the different cell types exhibit different biochemical characteristics or not.

Melancon et al. (1971) found a high histidase activity in epitheloid cells whereas hardly any activity was found in fibroblast-like cells. Cystathionine synthetase on the other hand is present in fibroblast-like cells but can hardly be detected in epitheloid cells (Uhlendorf & Mudd, 1968; Gerbie et al., 1972). Except those two enzymes Gerbie et al. (1972) found no significant differences in the specific activities of a number of other enzymes. Among them, the enzymes tested in appendix paper I. Separation of these two cell types was obtained by selective detachment of the fibroblast-like cells using trypsinization. In contrast, remarkable differences in the activity per cell was found for a variety of lysosomal enzymes between different cell groups isolated from the same culture, and analyzed by microchemical techniques (Galjaard et al., 1973, 1974[a-c]; Niermeijer et al., 1975; Kleijer et al., 1976).

The heterogeneity of three constitutive enzymes was studied in the different morphological cell types of amniotic fluid cell cultures and the results are described in appendix paper I. The characterization of the different cell types in the primary cultures was based on the description by Uhlendorf (1970); microchemical analyses were performed on two types of epitheloid cells (type I and II), fibroblast-like cells and large multinuclear amniotic fluid cells.

The activities of three lysosomal enzymes, α-glucosidase, β-galactosidase and β-hexosaminidase when expressed per cell appeared not to be significantly different in the small epitheloid and fibroblast-like cells; but 5 to 10 fold higher enzyme activities were found in the large multinuclear cells. To determine to what extent the enzyme activities per cell are related to cell size, the dry mass of the different cell types was measured by microinterferometry which allows the enzyme activities to be expressed per unit of dry mass. By performing enzyme assays on single cultured cells after interferometric

determination of the dry mass of the same cells we could show that the β-hexosaminidase activity per unit of dry mass was not significantly different in the various small amniotic fluid cell types and in the large cells.

Thus in prenatal diagnoses using conventional biochemical methods and amniotic fluid cell homogenates the morphological differences among various cell types in the amniotic fluid (sub-)cultures are not important in the case of the three enzymes tested. If, however, a prenatal diagnosis is based on microchemical analyses on groups of isolated freeze-dried cells in order to reduce the time interval between amniocentesis and diagnosis it is important to establish the nature of the prevailing cell types. The analysis of large multinuclear cells should either be avoided or comparisons should be made of cultures from a pregnancy at risk with similar cells in primary cultures of control samples.

2.1.2 Microchemical assays of β-galactosidase

To establish the reliability of microchemical assays on small groups of freeze-dried cells the results of such assays in three pregnancies at risk for G_{M1}-gangliosidosis were compared with those of conventional biochemical analyses on cell homogenate (appendix paper II). With the conventional methods the period needed to establish a diagnosis varied from $2\frac{1}{2}$ weeks (Lowden et al., 1973) to 4 weeks (Kaback et al., 1973; Booth et al., 1973). This period could be reduced to 12-20 days in the three cases described in appendix paper II by using assays on cell homogenate in microliter volumes (1-2 µl) and a further reduction to 9-12 days could be achieved by using microchemical assays of defined cell types, isolated in groups of 10-30 cells from freeze-dried clones of amniotic fluid cells. A good correlation was found between the results of microchemical assays on small numbers of cells and those of conventional analyses on homogenates. In each of the three pregnancies at risk for G_{M1}-gangliosidosis a non-affected child was predicted and the diagnoses were confirmed by the demonstration of a normal β-galactosidase activity in leucocytes after birth. Another approach to reduce the time interval between amniocentesis and the diagnosis could be a direct biochemical analysis of the

cell free amniotic fluid. However, no correlation between the β-galactosidase activity per ml of amniotic fluid and the outcome of the pregnancy was found. This observation is in agreement with data on other enzymes. It is now generally agreed that a reliable prenatal diagnosis requires the analysis of cultured amniotic fluid cells (Galjaard, 1980) except in a few cases where increased concentration of metabolites is a reliable parameter, for instance in methylmalonic aciduria (Morrow et al., 1970, 1977; Gompertz et al., 1974; Ampola et al., 1975; Mahoney et al., 1975; Nakamura et al., 1976).

2.2 Induction of differentiated gene expression

The idea that fusion of amniotic fluid cells with human G6Pase deficient hepatocytes from a patient with glycogenosis type I might lead to the induction of fetal G6Pase activity is described in chapter I. Until now we have not been able to activate in heterokaryons the G6Pase structural gene of amniotic fluid cells using the presumptive regulation mechanism of the liver cell.

As a model we first fused rat hepatocytes isolated by Dr. H.F. Bernard, (Dept. of Internal Medicine III, Erasmus University, Rotterdam; as described by Krenning et al., 1978) with either amniotic fluid cells or human diploid fibroblasts. However, the presence of rat G6Pase obscured the possible expression of the human G6Pase gene. Biochemical analysis of the fused cell populations and of mixed homogenates of both parental cell types did not show significant differences of G6Pase activity. Analysis of heterokaryons at the single cell level using a histochemical staining technique was unsuccessful because of the large variation in staining intensity among different hepatocytes.

In the fusion (figure 2, p.23) of human glycogenosis type I hepatocytes with amniotic fluid cells both parental cell types are G6Pase deficient and thus a small increase of G6Pase activity would be detectable. For this fusion the isolation of hepatocytes from a human liver biopsy was required and we tried different procedures to obtain viable hepatocytes which could be used in cell hybridization experiments. It was found that G6Pase activity in normal human hepatocytes was reduced to 15% during the first 3 days of cell cultivation. Therefore cell fusion would have to be carried out immediately after the

isolation of hepatocytes. By enzymic and mechanical treatment (according to methods described by Howard et al., 1973; Wands & Isselbacher, 1975) of a liver biopsy just enough viable hepatocytes were released for cell fusion (i.e. 200 000 cells) provided that the condition of the human liver was optimal. In the case of a liver biopsy from a patient with glycogenosis type I we were not able to recover sufficient viable cells for a successful cell fusion probably as a result of the large amount of stored glycogen. It was therefore not surprising that no induction of G6Pase could be demonstrated after fusing these hepatocytes with amniotic fluid cells.

In most somatic cell hybridization studies extinction of specialized functions has been observed after fusion of differentiated cells with non-differentiated cells (Ringertz & Savage, 1976; Deschatrette et al., 1979). There are, however, some recent examples of induction of differentiated gene products. Rankin & Darlington (1979) reported that human liver specific gene products, i.e. one or several serum proteins, were expressed in mouse hepatoma-human amniotic fluid cells. There was also a continuation of expression of mouse albumin and transferrin. From the results of studies on the expression of the albumin gene in cultured rat hepatoma cells, their dedifferentiated variants and in hybrids between the two types of cells, Cassio et al., (1981) suggested that suppression is caused by a block at the transcriptional level in the variants and in their somatic hybrids.

Despite the impressive advances in molecular biology our knowledge about the regulation of gene expression in mammalian cells is still very limited. We therefore chose to study gene expression at a more fundamental level before attempting to solve the technical problems encountered in our experiments on induction of gene expression in amniotic fluid cells.

2.3 Induction of constitutive gene expression

In the appendix paper III we studied the induction of expression of enzymes located in lysosomes, cytosol or in the endoplasmatic reticulum/Golgi complex by fusing chick erythrocytes which did not express these markers with mutant human fibroblasts that were deficient in the enzymes concerned. In the appendix papers IV and V the induction of

repair DNA synthesis is studied in the same hybrid system. The following section of this chapter was subdivided on the basis of the region of the cell in which the various markers studied are functional.

2.3.1 Lysosomal enzymes

Expression of α-glucosidase and β-galactosidase is induced in heterokaryons obtained after fusion of chick erythrocytes with human fibroblasts of patients with Pompe's disease and G_{M1}-gangliosidosis, respectively (appendix paper III).
Using immunological and electrophoretic methods it was shown that both enzymes are of chicken origin. The first moment that activity of α-glucosidase and β-galactosidase could be detected was around 4 days after fusion. Maximal activities were reached after about 15 days.

The minimal fusion index must be 15% heterokaryons, defined as the percentage of nuclei in heterokaryons of the total number of cell nuclei, in order to obtain reliable data from biochemical assays of homogenates after cell fusion. Reliable results can also be achieved if 10% of the total number of nuclei are erythrocyte nuclei in heterokaryons. Under special conditions of a low serum concentration and a confluent cell culture the fusion index can be maintained sufficiently high to study enzyme activities in heterokaryons during the first 15 days after fusion.

To extend the life-span of the heterokaryons and to inhibit proliferation of unfused cells DNA replication was suppressed by treating the human parental cells with mitomycin C before fusion. Linder et al. (1981) demonstrated that the reactivation process of the erythrocyte nucleus is not influenced by mitomycin C as judged from ^{3}H-uridine incorporation and reappearance of the nucleolus. A study on the influence of mitomycin C showed that the ratio of hydrolytic enzyme activity to fusion index was not influenced.

In the fusion of chick erythrocytes with fibroblasts from a patient with Sandhoff disease generation of β-hexosaminidase was not detected up to 23 days after fusion (appendix paper III). Conventional biochemical assays have, however, the disadvantage that low levels of β-hexosaminidase activity might be missed, because of admixture of hetero-

karyons with unfused parental cells. By using microchemical methods allowing the β-hexosaminidase assays in single heterokaryons this problem can be avoided. The activity of β-hexosaminidase was absent in heterokaryons even in those containing three or more erythrocyte nuclei and one human nucleus.

The expression of β-hexosaminidase activity was also studied in the fusion of chick erythrocytes with β-hexosaminidase A deficient fibroblasts from a patient with Tay-Sachs disease (appendix paper III). Since β-hexosaminidase B activity in the human mutant fibroblasts would obscure any expression of chick hexosaminidase, immunological methods had to be used. The immunological precipitation patterns of β-hexosaminidase in the human-chick heterokaryons, harvested at 2, 8, and 15 days after fusion were compared with the patterns of the human homokaryons by immunotitration with antiserum against human β-hexosaminidase. The antiserum used crossreacts with both β-hexosaminidase isozymes A and B and does not crossreact with chick β-hexosaminidase. We could not find any expression of chick β-hexosaminidase in Tay-Sachs fibroblast-chick erythrocyte fusion.

A limitation of this approach is that if chick hexosaminidase were to be induced but were immediately to form a heteropolymer with human subunits, the heteropolymeric molecule would be indistinguishable from homologous human enzyme and it would be precipitated in the same fashion. Such an explanation of the non-appearance of chick hexosaminidase can only be excluded by the use of an antiserum against chick β-hexosaminidase.

From the experiments described in appendix paper III it is interesting to note the differences in expression of the various lysosomal enzymes tested. The generation of α-glucosidase is rapid and efficient and it is quite exceptional that a level of activity of 140% of the normal enzyme level in chick fibroblasts is reached in heterokaryons 15 days after fusion. The generation of β-galactosidase is also detectable at 4 days after fusion but the maximum level of activity at about 2 weeks after fusion is only 20% of the normal activity in chick fibroblasts. As described above β-hexosaminidase activity was not induced at all. These differences in enzyme expression may have their origin at different levels of enzyme realization (Paigen, 1971, 1979).

A lower level or absence of enzyme expression can be due to insufficient transcription of the coding sequences and/or a restricted supply of protecting proteins leading to an increased turnover of the synthesized mRNA. Electron microscopical studies of the erythrocyte nuclei after fusion with rat L6 myoblasts suggested that erythrocyte nuclei are not fully reactivated at 5 days after fusion (Dupuy-Coin et al., 1976). It is possible that the differences in lysosomal enzyme expression are caused by different reactivation patterns for the sequences coding for the various enzyme polypeptide chains. Two studies in which it was suggested that the control of gene expression is at the transcriptional level have been discussed above, see p 26 (rRNA) and p 35 (albumin).

mRNA transport is another step in the complex pathway of enzyme realization which can be impaired, thus influencing enzyme expression. Evidence for a limitation in the amount of mRNA transport to the cytoplasm can be found in the work of Bramwell (1978). He demonstrated that only a small amount of chick ribosomal RNA appears in the cytoplasm of Hela-chick erythrocyte heterokaryons, although after the introduction of the chick nucleus in the heterokaryons a new polarization of endoplasmic reticulum and other organelles in the vicinity of the chick nucleus occurs (Dupuy-Coin et al., 1976; Hernandez-Verdun et al., 1979). A second explanation for the differences in induction of α-glucosidase, β-galactosidase and hexosaminidase may be that the transport out of the nucleus of the mRNAs concerned may differ.

Other explanations for the varying rates of appearance of the chick lysosomal enzymes in heterokaryons may be found at the translational and post-translational level. For instance the mRNAs for these enzymes must code for the characteristic hydrophobic leader sequences, which enable the precursor molecules to be transferred through the membrane into the intracisternal space of the endoplasmatic reticulum (Blobel, 1980). The induction of lysosomal enzymes may also be impaired by the absence or inadequacies of one of the various proteolytic and glycosylation steps required to form mature active enzymes from early precursor forms (Neufeld, 1981; Sly & Creek, 1981).

Our results from fusion experiments of chick erythrocytes with I-cell fibroblasts suggest that the glycosylation and mannose phos-

phorylation steps are adequately generated. The retention of both chick and human β-galactosidase in the heterokaryons suggests that chick N-acetylglucosamine-1-phosphate transferase is synthesized and is capable of restoring the deficient processing of human lysosomal enzymes. These results of appendix paper III indicate that this early step in the processing of lysosomal enzymes is not species specific.

Lysosomal enzymes have different biological half-lives which are the resultant of the relative rates of biosynthesis and degradation. The biological half-life of human β-galactosidase has been determined using an irreversible inhibitor of the enzyme; an average value of 10 days was found for the cultured fibroblasts (Van Diggelen et al., 1981). In hamster fibroblasts the half-life of β-glucuronidase has been reported to be 4 to 5 days while arylsulphatase A has a predicted half-life greater than 30 days (Strawser & Touster, 1980). For α-glucosidase the estimates obtained by feeding enzyme to human fibroblasts vary from 5-30 days depending on the source of enzyme (Reuser personal communication). The turnover of hexosaminidase was estimated to be 6-7 days (Hickman et al., 1974; Halley et al., 1978). If the half-lives of the chick lysosomal enzymes vary as much as those of the human enzymes, this can be an explanation for the observed differences in the rate of generation of lysosomal enzymes in chick erythrocyte human mutant fibroblast heterokaryons (appendix paper III).

Recent work on fibroblasts from patients with a combined β-galactosidase/neuraminidase deficiency has shown that the deficiency of β-galactosidase is due to enhanced degradation of an otherwise normal enzyme (Van Diggelen et al., 1981). This enhanced degradation appears to be due to the genetic deficiency of a 32 000 molecular weight protein which normally is required to protect β-galactosidase and probably neuraminidase from rapid interlysosomal degradation by the cell's own proteases (d'Azzo et al., 1982; d'Azzo, 1982). In principle it is thus possible that in chick erythrocyte-human fibroblast heterokaryons generation of lysosomal enzymes does occur but that some of these are rapidly degraded because of the absence of specific protective proteins inside the lysosome. This last processing step i.e. the binding of mature lysosomal enzyme to protective protein could be species specific and protection of chick lysosomal enzymes may not occur or may be in-

sufficient. This could, for example, be an explanation for the low chick β-galactosidase activity in heterokaryons from fusions of chick erythrocytes with G_{M1}-gangliosidosis fibroblasts. This aspect was further tested by fusing chick erythrocytes with human fibroblasts with a combined β-galactosidase/neuraminidase deficiency. Up to 8 days we could not find any β-galactosidase activity in the heterokaryons. This may be due to the fact that reactivation has not (yet) led to the presence of sufficient protecting protein of chick origin.

Along this line, we have tested whether the absence of generation of β-hexosaminidase after fusion of chick erythrocyte - human mutant fibroblasts could be explained by enhanced degradation in the post-translational steps of enzyme realization. Precursor form of chick β-hexosaminidase, obtained by ammonium chloride stimulation of chick fibroblasts was administered to Sandhoff fibroblasts and the ingested enzyme was found to be stable for at least 6 days. These results make it unlikely that the failure to produce significant levels of chick β-hexosaminidase activity was due to a rapid degradation of this enzyme.

A final explanation for the absence of generation of β-hexosaminidase in heterokaryons could be the fact that this enzyme consists of two different polypeptides (Srivastava and Beutler, (1973); Galjaard et al., 1974[c]; Thomas et al., 1974; for review see Mahuran & Lowden 1980) in contrast to β-galactosidase (Norden et al., 1974; Hoeksema, 1979) and α-glucosidase (Hasilik & Neufeld, 1980) which are initially synthesized as monomeric precursors. If this were also true for chick cells, the absence of generation of hexosaminidase activity in heterokaryons might be due to an impairment for the coordinated association of subunits necessary to obtain full activity.

Although we have not been able to verify with certainty the exact cause of the differences in the induction of lysosomal enzymes in our experiments, the chick erythrocyte - human mutant fibroblast heterokaryons provide an attractive model to follow biochemically and ultrastructurally the intracellular route of "de novo" synthesized enzymes and to study the species specificity of the various post-translational modification steps.

2.3.2 *The cytosolic enzyme glucose-6-phosphate dehydrogenase*

Similar studies to those described above have been carried out with the cytosolic enzyme glucose-6-phosphate dehydrogenase (G6PD) is studied. During a period of 30 days no increase of G6PD activity was detected after fusion of chick erythrocytes with two different G6PD deficient cell strains with residual activities of 4% and 20%, respectively. This absence of induction of G6PD activity may find its explanation at different levels of enzyme realization. For a discussion of the problems at the transcriptional level and the transport of mRNA see p 38 . Since recently cDNA of G6PD has been made (Persico et al., 1981) it will be possible to investigate whether in chick-human heterokaryons expression of mRNA for G6PD is present or absent.

2.3.3 *DNA repair in the cell nucleus*

2.3.3.1 *Restoration of DNA repair synthesis*

The induction of chick DNA repair functions was investigated by the analysis of the DNA repair capacity after exposure to ultraviolet irradiation of heterokaryons obtained after fusion of chick erythrocytes with either normal human fibroblasts (appendix paper V) or cells from patients with xeroderma pigmentosum (XP) belonging to complementation groups A, B, C and D (appendix paper IV). In all 4 XP complementation groups studied DNA repair activity in human nuclei occurred after fusion with chick erythrocytes, albeit with different kinetics. In fusions with XP-A cells unscheduled DNA synthesis (UDS) is already evident after one day and reaches almost the same level found in normal human nuclei after 5-8 days. Increased levels of UDS are found in the human nuclei of the B, C and D groups at 5 days after fusion. At 8 days these UDS levels are about 50% of that found in normal human nuclei. In the chick nuclei of the heterokaryons which initially do not show DNA repair the pattern of UDS parallels that of the human counterpart. The maximum levels reached in chick nuclei are, however, about one third of those in human nuclei.

The results described in appendix paper IV are in contrast with data published by Darzynkiewicz et al. (1972) who did not find any induction of DNA repair at one and seven days after fusion. At the

time of their experiments these authors did not know anything about the type of mutation involved. It is now known that most of the excision-deficient XP strains belong to the complementation groups A, C and D. It is possible that the cell strain used by Darżynkiewicz and coworkers belongs to the XP groups C or D, with a low rate of DNA repair restoration which may have escaped detection under their experimental conditions.

In order to investigate the interaction between human and chick repair functions chick fibroblasts were fused with fibroblasts of XP group A and C. The normal UDS levels in chick fibroblast nuclei as measured by autoradiography after ultraviolet irradiation is about 40-50% of that in human fibroblasts, which is in agreement with results by Wade et al. (1980) who used excision of dimers as a parameter. In the chick fibroblasts-XP group A heterokaryons restoration of UDS in the human nuclei also occurs rapidly. Within the first 24 hours the UDS level is comparable with the level in normal human-chick fibroblast heterokaryons. In heterokaryons obtained after fusion of XP group C cells with chick fibroblasts DNA repair remains at the level of that in chick cells. This finding suggests that the 'chick factor' responsible for the restoration of the defect in XP group C is rate limiting in the heterokaryons. In fusions with chick erythrocytes the DNA repair restoration is about the same for the XP groups B, C and D which suggests that also in the restoration of XP groups B and D the 'chick factors' are rate limiting.

The early start of DNA repair restoration in XP group A cells after fusion with chick erythrocytes indicates that the responsible 'chick factor' must be present at an early stage of reactivation of the chick nuclei. Since the modification of the chromatin structure is one of the first steps in the reactivation it may be that one of the factors involved in this process is defective in XP group A. The rapid synthesis of such a factor during chick nuclei reactivation may also facilitate the accessibility of the human DNA to repair enzymes. Such a defect in the modification of the chromatin structure in XP group A has been postulated previously by Mortelmans et al. (1976), although repetition of the experiments of Mortelmans et al. have not been successful so far.

2.3.3.2 Suppression of DNA repair synthesis

DNA repair synthesis is influenced by still another chick component. In the control human fibroblast-chick erythrocyte heterokaryons the UDS in the human nuclei is suppressed from 24 hours after fusion on. This suppression is stronger when the ratio of erythrocyte nuclei to human nuclei in the heterokaryons increases. The maximal effect occurs at about 36 hours after fusion (appendix paper V).

Various studies on heterokaryons after fusion of chick erythrocytes and different types of mammalian cells indicated a reduced RNA synthesis in the latter (Carlsson et al., 1973; Darżynkiewicz et al., 1974[a,b]). In order to investigate a possible relationship between the suppression of UDS and an inhibition of RNA synthesis, (^{3}H)uridine incorporation was determined at different time intervals after fusion of chick erythrocytes with either human fibroblasts or Hela cells (appendix paper V). In Hela cells the amount of incorporated (^{3}H)-uridine as measured by autoradiography was found to be about 10 times higher than that in normal human diploid fibroblasts. During the first 24 hours after fusion normal levels of DNA repair were observed in the nuclei of normal human fibroblasts, whereas RNA synthesis was markedly inhibited. This suggests that during this period the DNA repair capacity in these nuclei is independent of RNA synthesis. This assumption is in agreement with the results of cybridization experiments in which the repair functions are maintained in the cytoplasts in the absence of RNA synthesis for about 20 hours (Keijzer et al., 1982 in press).

From 24 hours after fusion with chick erythrocytes the UDS decreases in the human fibroblast nuclei and reaches a minimum after about 36 hours. The minimum level is lower when relatively more erythrocyte nuclei are present in a heterokaryon. The incorporation of (^{3}H)uridine increases from 24 hours on and reaches a maximum at 36 hours; the maximum level of RNA synthesis of chick-human heterokaryons is reduced compared to that in the unfused human fibroblasts; again the reduction is stronger when relatively more erythrocyte nuclei are present (appendix paper V). These findings suggest a correlation between the suppression of DNA repair in human fibroblast nuclei and

a reduction of RNA synthesis. The latter is likely to result in a diminuation of repair enzymes which are normally required to maintain normal DNA repair. In fusions of chick erythrocytes with HeLa cells no reduction of UDS occurs (Darżynkiewicz et al., 1972; appendix paper V) despite a suppression of RNA synthesis. This can be explained by a sufficiently high residual RNA synthesis (appendix V) allowing for adequate amounts of repair enzymes.

Carlsson et al. (1973) and Darżynkiewicz et al. ($1974^{a,b}$) suggested previously that suppressors of RNA synthesis, ordinarily confined to the chick nucleus, are released with subsequent redistribution among all the nuclei of the heterokaryons. These suppressors may be hydrolysed either in transit between the nuclei or within the nuclei by intracellular proteases, after which RNA synthesis increases again. Nuclear protease(s) tightly associated with chromatin and with high specificity for individual histon fractions has been described by Bartley & Chalkley (1970) and Gareles et al. (1972). Because inhibitors of these proteases, N-α-tosyl-L-lysyl-chloromethane and N-α-tosyl-L-arginine methyl ester suppress the reactivation of chick-erythrocytes, Darżynkiewicz et al. ($1974^{a,b}$) postulated that nuclear proteases might be involved in the regulation of transcription via hydrolysis of suppressors.

In the XP group A-chick erythrocyte heterokaryons an early restoration of the UDS in detectable (appendix paper IV) at the same time that suppression of the RNA synthesis occurs, which in control human fibroblasts resulted in suppression of UDS. That reactivation as well as suppression occur also in the XP group A-chick heterokaryons appeared from the reverse gene dosage effect: in heterokaryons with one extra human nucleus the relative amount of suppressors is lower of redistribution among the three nuclei and in these heterokaryons a higher level of UDS is detectable (appendix paper IV). These experiments demonstrate clearly the difference in kinetics of DNA restoration between XP group A and XP group B, C and D, indicating that different gene products are involved in the genetic defect of the A group on one side and the B,C and D group on the other. This conclusion is also supported by the study of complementation kinetics in XP-XP heterokaryons performed by Pawsey et al. (1979) and Matsukuma et al.

(1981).

The molecular nature of the defective factors in the different types of XP has not yet been elucidated. The cell fusion studies have so far only provided indirect evidence that they represent different gene products. At the present time two approaches can be followed to solve this problem. Firstly, experiments in which microbial repair enzymes have been introduced in XP cells, have shown that the XP defects can be corrected (phenotypic correction)(Tanaka et al., 1975, 1977). In as yet unpublished experiments de Jonge and Hoeijmakers in our laboratory have shown that phenotypic correction of the XP-A defect can be achieved by microinjection of extracts of normal human cells directly into the cytoplasts of XP-A cells. This microinjection system may provide a test system for purified protein fractions from wild-type human cells which finally may result in the biochemical characterization of the XP-A factor.

A second approach, which is now followed in different laboratories, is the isolation and cloning of the genes coding for XP factors. These experiments require the correction of the XP defect by introduction of wild-type human DNA fragments (genotypic correction). In the last few years rapid progress has been achieved in DNA transformation of mammalian cells (e.g. Wigler et al., 1977). Recently, Japanese investigators have been able to correct the XP-A defect after transfection with wild-type DNA fragments (Tanako et al., 1982). The principle techniques are available to rescue the transforming DNA fragments from these corrected XP cells using the recombinant-DNA technology. When these fragments have been isolated, their function in the DNA repair pathway can be studied in in vivo as well as in in vitro transcription and translation systems.

SUMMARY

In this thesis two aspects of gene expression in cultured cells have been studied: the heterogeneity in gene expression in relation with the development and application of microchemical techniques for the prenatal diagnosis of inborn errors of metabolism and the possibility of inducing gene expression in cultured cells using somatic cell hybridization.

The (prenatal) diagnosis of genetic metabolic diseases is based on a comparison of data after biochemical analysis of cultured amniotic fluid cells and fibroblasts from normal individuals, heterozygotes and affected patients or fetuses. For a reliable prenatal diagnosis the effect of cell cultivation conditions and various morphological cell types within one culture on the biochemical parameters to be studied must be known. In this thesis the activity of three lysosomal enzymes, acid α-glucosidase, β-galactosidase and β-N-acetylhexosaminidase were investigated in relation to different cell types in primary amniotic fluid cell cultures. This was possible by microchemical analysis on small groups (10 - 100) of morphologically different clones isolated from freeze-dried amniotic fluid cell cultures. The activities of the three lysosomal enzymes when expressed per cell appeared to be about the same in the small epithelioid cell types 1 and 2 and the fibroblast-like cells. In the large, often multinuclear cells however, the enzyme activity per cell was 5 to 10 fold higher than this in the other cell types. By performing combined microinterferometric determinations of the dry mass and enzyme assays on single cells we were able to show that no differences in lysosomal enzyme activities exist between the various amniotic fluid cell types if the activity is expressed per unit dry mass (appendix paper I).

The microchemical assays on defined amniotic fluid cell types were applied in the prenatal monitoring of three pregnancies at risk for G_{M1}-gangliosidosis. Comparison with conventional biochemical analysis showed the reliability of the microchemical techniques used. Application of the latter allows the time interval between amniocentesis and prenatal diagnosis to be shortened to 9-12 days (appendix paper II).

Since only about eighty out of a total of more than 200 known genetic enzyme deficiencies are expressed in cultured cells several at-

tempts have been made to widen the scope of prenatal diagnosis. The two main approaches are direct analysis of DNA and the induction of gene expression by somatic cell hybridization. Our attempts to induce glucose-6-phosphatase, a liver enzyme deficient in glycogenosis type I (Von Gierke's disease), by fusion of cultured fibroblasts and amniotic fluid cells with hepatocytes were unsuccessful. Although the main reasons for the failure were of a technical nature we felt there was inadequate knowledge about the regulatory mechanisms involved in the induction of gene expression. The experimental work described in appendix papers III, IV and V deals with the study of the induction of enzymes located in different subcellular compartments using hybridization of human mutant fibroblasts with chick erythrocytes.

Xeroderma pigmentosum (XP) is an autosomal recessive skin disease based on defective DNA repair synthesis after ultraviolet irradiation. Somatic cell hybridization studies have so far revealed 7 complementation groups (A to G) among the patients investigated. After fusion of XP-A fibroblasts with chick erythrocytes restoration of unscheduled DNA synthesis (UDS) in the human nuclei was observed within the first day after fusion. Near normal levels of UDS were reached after 5-8 days. DNA repair, absent in mature chick erythrocyte nuclei is reactivated in heterokaryons at a manor similar to that of the restoration in human nuclei although at a lower level. These experiments (appendix paper IV) indicate that the reactivated chick genome generates a factor which is capable of a fast restoration of the defective DNA repair in XP-A. The kinetics of restoration of UDS in fusion with XP fibroblasts from complementation groups B, C and D are clearly different from that in fusions with XP-A nuclei. Restoration of human DNA repair is not measured before 4 days after fusion and only 50% of normal UDS levels is reached at 8 days after fusion. These differences are also observed for chick fibroblast-XP heterokaryons. The factors coded for by the chick genome apparently are rate limiting in the restoration of the genetic defects involved in XP-groups B, C and D.

In the control human fibroblast-chick erythrocyte heterokaryons suppression of UDS in the human nuclei is detectable between 1-5 days after fusion, with a maximal effect around 36 hours after fusion. The extent and duration depends on the ratio of erythrocyte nuclei to human nuclei in the heterokaryons. Evidence is presented that this

suppression is due to decreased levels of enzymes involved in UDS as a result of inhibition by chick components of human RNA synthesis.

In a next set of hybridizations human fibroblasts derived from patients with glycogenosis II (α-glucosidase deficient), G_{M1}-gangliosidosis (β-galactosidase deficient), Tay Sachs and Sandhoff types of G_{M2}-gangliosidosis (β-hexosaminidase A deficient and β-hexosaminidase A and B deficient, respectively) were fused with chick erythrocytes. In heterokaryons the generation of the lysosomal enzymes α-glucosidase and β-galactosidase is first detectable at 4 days after fusion and maximum levels are obtained after 15 days. Both enzymes appear to be of chick origin. The maximal level of activity in heterokaryons is lower for β-galactosidase than for α-glucosidase (20% and 140%, respectively, of normal chick fibroblast activities). Unexpectedly, β-hexosaminidase showed a different behaviour than the other lysosomal enzymes tested since no generation of activity could be demonstrated up to 23 days after fusion.

Explanations for the differences in the induction of gene expression are discussed for all levels of enzyme realization. Recently, it has become more and more clear that the formation of mature, active lysosomal enzymes requires not only the synthesis of polypeptide chains but also their processing involving a complex pathway of post-translational events. We have investigated an early step in this processing, i.e. the phosphorylation of mannose in high mannose oligosaccharides of (lysosomal) glycoproteins in the ER/Golgi complex which is deficient in I-cell disease. Fusion of fibroblasts from such a patient with erythrocytes resulted in a retention of both human and chick β-galactosidase which is an indirect demonstration of the generation of chick N-acetylglucosamine-1-phosphate transferase. Interestingly, this chick enzyme seems to be able to perform one of the early processing steps of human lysosomal enzymes as well.

Although we have not been able to verify the exact cause of the observed differences in the induction of gene expression, the chick erythrocyte-human mutant fibroblast heterokaryons provide an attractive model to follow biochemically and ultrastructurally the intracellular route of 'de novo' synthesized enzymes and to study the species specificity of various steps in enzyme realization.

SAMENVATTING

In dit proefschrift zijn twee facetten van genexpressie in gekweekte cellen bestudeerd, de heterogeniteit van genexpressie in verband met de ontwikkeling en toepassing van microchemische technieken voor prenatale diagnostiek van aangeboren afwijkingen en de mogelijkheid tot inductie van genexpressie in gekweekte cellen met behulp van somatische celhybridisatie.

De (prenatale) diagnose van erfelijke stofwisselingsstoornissen is gebaseerd op een vergelijking van gegevens na biochemische analyse van gekweekte vruchtwatercellen en fibroblasten van gezonde individuen, heterozygoten en patienten of aangedane foetussen.
Voor een betrouwbare prenatale diagnose moet de invloed van celkweek condities en van de morphologisch verschillende celtypes binnen een celkweek op de bestudeerde biochemische parameters bekend zijn. Daarom is de activiteit van 3 lysosomale enzymen, zure α-glucosidase, β-galactosidase en β-N-acetyl-hexosaminidase onderzocht in de verschillende celtypen van primaire vruchtwaterkweken.
Tijdens de microchemische analyse is het mogelijk om kleine kloontjes gevriesdroogde cellen morfologisch te herkennen en een nauwkeurig geteld aantal cellen te isoleren. De activiteit van de drie lysosomale enzymen per cel is niet duidelijk verschillend in de kleine epitheloïde celtypes I en II en in het fibroblastachtige celtype. Een 5 tot 10 keer hogere activiteit per cel wordt gevonden in de grote, meestal meerkernige cellen. Het gecombineerd toepassen van microinterferometrische bepalingen van de droge massa en enzym metingen op dezelfde (enkele) cel toonde aan dat er geen verschil in lysosomale enzym activiteit bestaat tussen de verschillende celtypen in een vruchtwaterkweek als de activiteit per eenheid van droge massa wordt uitgedrukt (publikatie I).

De microchemische analyse op de gedefinieerde typen vruchtwatercellen werd toegepast bij prenatale diagnostiek tijdens 3 zwangerschappen met een verhoogd risico voor G_{M1}-gangliosidosis. De vergelijking met de conventionele biochemische methoden toonden de betrouwbaarheid van de microchemische technieken aan. Tevens werd door gebruik te maken van deze micromethode de tijdsduur tussen de vrucht-

waterpunctie en de diagnose terug gebracht tot 9-12 dagen (publikatie II).

Omdat slechts ongeveer 80 van de totaal ruim 200 nu bekende erfelijke enzymafwijkingen in gekweekte cellen tot expressie komen, zijn verschillende pogingen ondernomen om de mogelijkheid van prenatale diagnostiek uit te breiden. De twee belangrijkste benaderingswijzen zijn de direkte analyse van het DNA en de inductie van genexpressie met behulp van somatische celhybridisatie. Onze pogingen om glucose-6-phosphatase, een lever enzym deficient in glycogenosis type I (de ziekte van Von Gierke) te induceren door fibroblasten en vruchtwatercellen te fuseren met hepatocyten hadden geen succes, mede door moeilijkheden van technische aard. In het algemeen kan worden gesteld dat voor toepassing van inductie van genexpressie in de prenatale diagnostiek meer inzicht in de regulatie mechanismen van genexpressie in eukaryote cellen moet worden verkregen.

Een poging hiertoe bij te dragen is beschreven in de publikaties III, IV en V, waarin de inductie van enzymen, gelocaliseerd in verschillende celorganellen wordt bestudeerd door middel van hybridisatie van huidfibroblasten van patienten met erythrocyten van de kip. Deze erythrocyten vertonen vrijwel geen DNA en RNA synthese, zodat na fusie de invloed van het fibroblast genoom op de inductie van genexpressie in de kippekern kan worden onderzocht. De kernen van de erythrocyten zijn ook na fusie morfologisch duidelijk verschillend van de menselijke kernen, waardoor een goed onderscheid tussen homo- en heterokaryons mogelijk is. De genen, die in dit onderzoek zijn bestudeerd, zijn betrokken bij het DNA herstelproces, essentieel voor de handhaving van erfelijke informatie, of bij het realisatieproces van functionele lysosomale enzymen.

Xeroderma pigmentosum (XP) is een autosomale recessieve huidaandoening als gevolg van een defect in het herstel van DNA beschadigingen, die door zonlicht worden veroorzaakt in de cellen van de huid. Met behulp van somatische celhybridisaties is aangetoond dat tenminste 7 verschillende gendefecten verantwoordelijk kunnen zijn voor dit syndroom (complementatie groepen A-G). Een maat voor het DNA herstel is de hoeveelheid (^{3}H) thymidine die in het DNA wordt ingebouwd na bestraling met ultraviolet licht (unscheduled DNA synthesis; UDS). Na fusie

van XP-A fibroblasten met erythrocyten van de kip werd herstel van UDS in de menselijke kern op de eerste dag na fusie waargenomen. De UDS bereikte in 5-8 dagen vrijwel het controle niveau. In rode bloedcelkernen van de kip, die voor fusie geen DNA-herstelsynthese vertonen, vond ook reactivatie van UDS plaats na fusie, maar op een lager niveau. Deze experimenten (publikatie IV) geven aan dat reactivatie van bepaalde genen in de erythrocyten van de kip een factor kan opleveren die in staat is tot een snel herstel van het defecte DNA repair in de xeroderma celkern. De kinetiek van het herstel van UDS in XP fibroblasten uit de complementatiegroepen B, C en D is duidelijk verschillend van die in de XP-A kernen na fusie met erythrocyten van de kip. Herstel van het menselijke DNA repair wordt niet voor de 4e dag na fusie gevonden en slechts 50% van het controle UDS niveau wordt bereikt op de 8e dag na fusie. Deze verschillen zijn ook in de kippefibroblast-XP heterokaryons waargenomen. De factoren gecodeerd door het genoom in de kip zijn blijkbaar snelheidsbepalend in het herstelproces van de erfelijke afwijkingen welke betrokken zijn bij XP groep B, C en D.

Naast de invloed van het fibroblast genoom op de inductie van genen betrokken bij het DNA herstelproces, zijn er factoren uit de kip die remmend werken op de UDS in de menselijke kern. In de heterokaryons gevormd door normale fibroblasten van de mens en erythrocyten van de kip is onderdrukking van UDS in de menselijke kern tussen 1 - 5 dagen na fusie waarneembaar. Het maximale effect ligt rond 36 uur na fusie. Omvang en duur van suppressie zijn afhankelijk van de verhouding van het aantal kippekernen en menselijke kernen in de heterokaryons. We hebben aanwijzingen dat deze suppressie veroorzaakt wordt door een verlaagd niveau van enzymen, die betrokken zijn bij UDS als gevolg van factoren uit de kippecel die remmend werken op de RNA synthese in de menselijke kern.

De inductie van genexpressie van de eerdergenoemde lysosomale enzymen is bestudeerd in de heterokaryons waarin menselijke fibroblasten afkomstig van patienten met glycogenosis II (α-glucosidase deficiëntie), G_{M1}-gangliosidosis (β-galactosidase deficiëntie), Tay Sachs en Sandhoff type van G_{M2}-gangliosidosis (β-hexosaminidase A respectievelijk β-hexosaminidase A en B deficiënties) zijn gefuseerd met erythrocyten van

de kip. In de heterokaryons is het genereren van de lysosomale enzymen α-glucosidase en β-galactosidase voor het eerst zichtbaar na 4 dagen met een maximale activiteit 15 dagen na fusie. Beide enzymen blijken afkomstig van de kip te zijn. Het uiteindelijke activiteitsniveau in heterokaryons is lager voor β-galactosidase (20% van de controle activiteit in kippefibroblasten) dan voor α-glucosidase (140% in vergelijking met kippefibroblastèn niveau). β-hexosaminidase had onverwachts een ander gedragspatroon dan de andere geteste enzymen, daar geen enzym activiteit kon worden aangetoond tot en met 23 dagen na fusie.

Verklaringen voor de verschillen in inductie van genexpressie worden besproken voor alle stappen die leiden tot de realisatie van een functioneel enzym. Recent onderzoek heeft uitgewezen dat voor de vorming van volledig actieve lysosomale enzymen niet alleen de synthese van polypeptide ketens nodig is, maar dat deze daarna nog onderhevig zijn aan een reeks van post-translationele modificaties. We hebben een van de stappen in het modificatieproces onderzocht, namelijk de fosforylering van mannose in 'high mannose' oligosacchariden van (lysosomale) glycoproteïnen. Deze fosforyleringsstap vindt plaats in het endoplasmatisch reticulum/Golgi complex en is deficient in 'I-cell disease'. Fusie van fibroblasten afkomstig van deze patienten met erythrocyten resulteerde in intracellulaire β-galactosidase activiteit, afkomstig van zowel de mens als de kip, waarmee indirect wordt aangetoond dat het voor deze stap verantwoordelijke enzym van de kip, N-acetyl glucosamine-1-fosfaat transferase, wordt gereactiveerd. Interessant is dat dit enzym ook blijkt te functioneren in het modificatie proces van menselijke lysosomale enzymen.

Hoewel we niet in staat zijn geweest om een exacte oorzaak aan te geven van de waargenomen verschillen in de inductie van genexpressie, hebben deze experimenten laten zien dat de fusie van mutant fibroblasten van de mens met erythrocyten van de kip een zeer aantrekkelijk studie object is om biochemisch en ultrastructureel de intracellulaire route van de nieuw gesynthetiseerde enzymen te volgen en de soort specificiteit van de verschillende stappen van het enzym realisatieproces te bestuderen.

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NAWOORD

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Delft, april 1982

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appendix papers I-V

paper 1

Lysosomal Enzyme Activities in Different Types of Amniotic Fluid Cells Measured by Microchemical Methods, Combined With Interference Microscopy

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Summary. In primary amniotic fluid cultures, four distinct types of cells were characterized as epithelioid (E I and E II), fibroblast-like (F), and large cells. Small numbers (1—200) of freeze-dried cells were isolated from colonies of each cell type and analyzed for the activity of three lysosomal enzymes: β-N-acetylglucosaminidase, β-galactosidase, and α-glucosidase.

When expressed per cell, the activities for each of the enzymes were not significantly different among the small types of cells (E I, E II, and F). However, 5 to 10-fold higher enzyme activities were found in the large cells. The dry mass of individual large cells, as measured by microinterferometry, was also 5 to 10 times higher than that of the smaller cell types.

When expressed per unit of dry mass, the enzyme activities tested, appeared to be independent of the type of amniotic fluid cell. The significance of this observation for the rapid prenatal diagnosis of metabolic diseases is discussed.

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Introduction

At present the prenatal detection of about 50 genetic metabolic disorders is possible by the measurement of a specific enzyme deficiency in cultured amniotic fluid cells (Burton et al., 1974; Milunsky, 1975). To reduce the time interval between amniocentesis and diagnosis, micromethods enabling the analyses of small groups of freeze-dried cells (50—100 cells) have been successfully applied (see for review, Galjaard et al., 1975). In the prenatal analysis of glycogenosis Type II (Galjaard et al., 1973; Niermeijer et al., 1975), Fabry's disease (Galjaard et al., 1974), and G_{M1}-gangliosidosis (Kleijer et al., 1976), remarkable differences in enzyme activities per cell were sometimes found between different cell groups isolated from the same culture. Such a heterogeneity might be the result of the presence of different types of amniotic fluid cells with different enzyme activities.

In amniotic fluid cultures several types of cells have been defined on the basis of their morphology (Uhlendorf, 1970; Sutherland et al., 1974; Hoehn et al., 1974). Besides the most common types of epithelioid and fibroblast-like cells, different types of large, often multinuclear, cells have been described.

Melancon et al. (1971) and Gerbie et al. (1972) found no significant differences in the specific activities of a number of enzymes in subcultures of epithelioid and fibroblast-like amniotic fluid cells. Separated cultures of these two cell types were obtained by selective detachment of the fibroblast-like cells using trypsinization. So far no biochemical analyses have been reported on different cell types in primary cultures of amniotic fluid cells. This seemed important to us for a correct interpretation of data obtained by microchemical assays of such cultures.

In the present study we have investigated the activities of enzymes in different morphologic cell types that were isolated by microdissections under microscopic control from primary cultures as described earlier (Galjaard et al., 1973, 1975). The activities of β-galactosidase, β-N-glucosaminidase, and α-glucosidase were analyzed in sub-microliter volumes of 4-methylumbelliferyl substrate and the fluorescence was measured by microfluorometry (Galjaard et al., 1973, 1975).

To determine to what extent the enzyme activities per cell are related to cell size, the dry mass of different cell types was measured by microinterferometry and the enzyme activities per unit of dry mass were determined. The implications of varying proportions of different cell types in primary amniotic fluid cultures for the reliability of prenatal diagnosis of metabolic diseases are discussed.

Materials and Methods

Cultivation and Isolation of Amniotic Fluid Cells. Amniotic fluid samples were obtained by transabdominal amniocentesis in the 14th—20th week of pregnancies that were either monitored for chromosomal aberrations or terminated for nongenetic reasons. The amniotic fluid cells were cultured as described earlier (Niermeijer et al., 1975), and the various cell types and the topography of the colonies were examined with an inverted phase-contrast microscope after 9—15 days of growth.

For microchemical enzyme assays, primary cultures of amniotic fluid cells were grown on dishes with a thin plastic bottom, and freeze-dried as soon as a sufficient number of colonies was

available. For each specific cell type present in the culture 10—40 groups of cells were isolated according to methods described by Galjaard et al. (1973, 1975). Each dissected piece of plastic foil contained 1—10 cells for the assay of β-N-acetylglucosaminidase, 10—20 cells for β-galactosidase, and 70—200 cells for α-glucosidase. The exact number of cells was usually counted after isolation, but in the case of confluent epithelioid colonies the total cell number per colony was counted before freeze-drying.

Microchemical Assays of Enzyme Activities. The pieces of plastic foil with counted numbers of freeze-dried cells were incubated under hexadecane-paraffin oil (40/60 vol/vol) for 1 h in 0.3—1 µl of the appropriate substrate solution, the exact volume depending on the size of the dissected pieces of plastic foil. The reaction was stopped by addition of 3—5 µl of 0.5 M sodium carbonate buffer, pH 10.7. The amount of liberated 4-methylumbelliferone in the microdroplets was measured in capillary tubes, using a Leitz MPV microspectrofluorometer. Details of the procedures used have been described elsewhere for the assay of β-N-acetylglucosaminidase (Reuser et al., 1976), β-galactosidase (Galjaard et al., 1975; Kleijer et al., 1976), and α-glucosidase (Galjaard et al., 1975).

Determination of the Dry Mass of Single Cells. The dry mass of single cultured amniotic fluid cells of various types was determined with a Leitz double-beam interference microscope, using a 20 X objective, a 6.3 X ocular, and scanning equipment according to de Josselin de Jong et al. (1973).

After 6—8 days of growth on coverslips the cultures were immersed in 0.9% NaCl and the optical path differences were measured over the whole area of separate cells. The automatically integrated value of the optical path difference was used as relative measure for the dry mass of the cell (de Josselin de Jong et al., 1973; Caspersson et al., 1962, 1965; Sandritter et al., 1960).

In combined microchemical and interferometric analyses of the same cell the dry mass was determined on single freeze-dried cells, which were immersed in heptane. After evaporation of heptane the enzyme activities were measured as described before.

Results

Characterization of Cell Types in Primary Amniotic Fluid Cell Cultures. Throughout the primary cultures of amniotic fluid cells, four cell types were distinguished on the basis of their morphology and of the characteristics of the colonies:

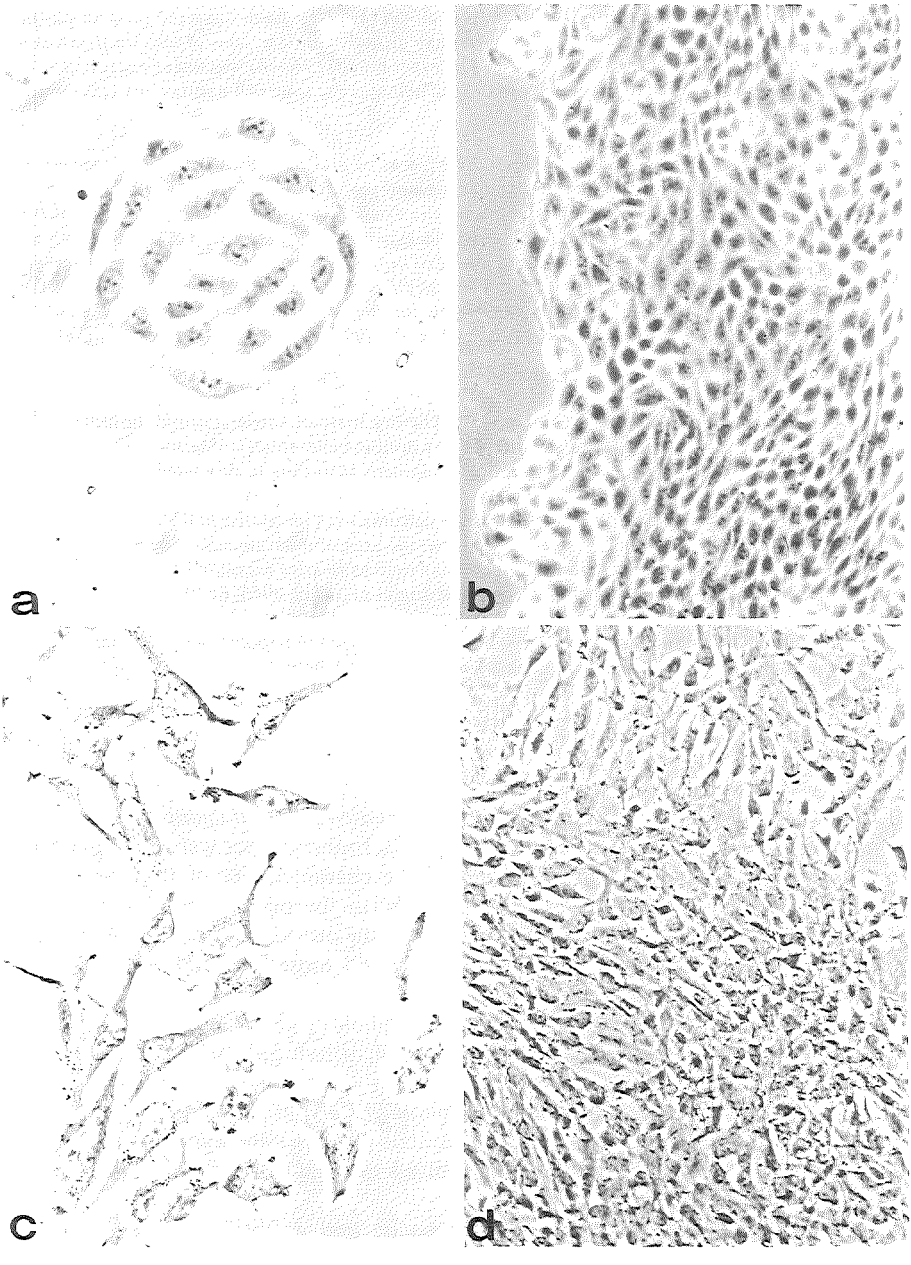
E I—Epithelioid cells, growing in 'islands' in the primary culture. In early clonal growth, cell proliferation is seen only at the outside of a colony. At a later stage, groups of proliferating cells are observed more randomly through the colony (Fig. 1 a and b).

E II—Epithelioid cells, with a similar morphology as the E I type, but which do not grow in islands. Proliferating cells are distributed at random through the colony (Fig. 1 c and d).

F—Fibroblast-like cells, with a morphology like human skin fibroblasts. In early clonal growth, cells with long cytoplasmic processes are seen; at a later stage of cultivation the typical pattern of fibroblasts running close and parallel to each other is observed (Fig. 1 e and f).

L—Large cells, with one or more nuclei and a fibrillar-like cytoplasm. Although these cells were observed in almost all primary amniotic fluid cultures, the largest proportions occurred in poorly growing cultures (Fig. 1 g).

The frequency of the different cell types in different cultures was highly variable, even if the cultures originated from the same amniotic fluid sample.


a
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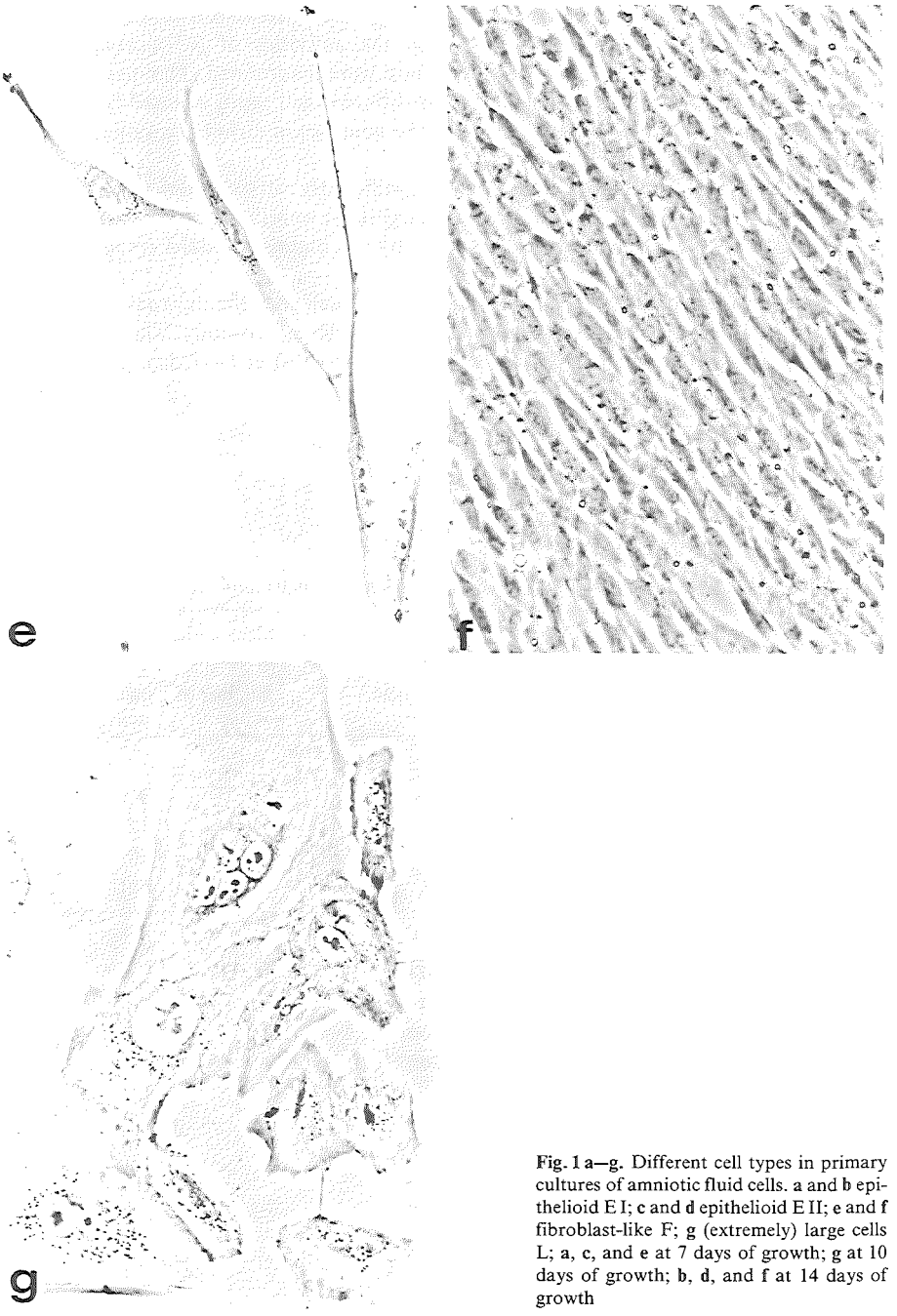

Fig. 1 a—g. Different cell types in primary cultures of amniotic fluid cells. a and b epithelioid E I; c and d epithelioid E II; e and f fibroblast-like F; g (extremely) large cells L; a, c, and e at 7 days of growth; g at 10 days of growth; b, d, and f at 14 days of growth

Lysosomal Enzyme Activities and Dry Mass per Cell. Groups of cells, which were homogeneous with respect to their morphology, were isolated from the colonies present after 9—15 days of growth, and the activities of β-N-acetylglucosaminidase, β-galactosidase, and α-glucosidase were determined. The mean activities expressed per cell were calculated for different cell types isolated from one single culture. These results and those of the analyses in different amniotic fluid cell cultures are summarized in Table 1.

The enzyme activities per cell were not significantly different in the three small cell types (E I, E II, and F). Those in the large cells were, however, much higher and also more heterogeneous, as is shown by the higher standard errors of the mean.

Since the enzyme activities might be related to cell size, the dry mass of single cells of each cell type was measured with interference microscopy. The results of dry mass measurements of 137 cells of different types after 6—8 days of growth

Table 1. Lysosomal enzyme activities in different cell types of primary amniotic fluid cell cultures

Cell type	Mean activities within individual cultures			Mean activities of different cultures	
	Number of cell groups	Mean activity[a]	S.E.M.[b]	Number of cultures	Mean activity[a]
β-N-acetylglucosaminidase					
Epithelioid I	1	103	—	2	123
Epithelioid II	50	109	7	4	123
Fibroblast-like	20	85	9	4	107
Large	26	545	66	4	501
β-galactosidase					
Epithelioid I	10	11.2	0.8	4	8.5
Epithelioid II	25	11.0	0.6	7	6.8
Fibroblast-like	8	7.9	0.8	7	6.7
Large	6	58.2	26.7	7	43.6
α-glucosidase					
Epithelioid I	3	0.15	0.04	1	0.15
Epithelioid II	17	0.19	0.02	2	0.20
Fibroblast-like	17	0.25	0.07	2	0.23
Large	18	2.67	0.59	2	2.45

[a] Moles 4 MU/h/cell $\times 10^{-14}$

[b] Standard error of the mean

are presented in Figure 2. The dry mass of the small cell types, i.e., E I, E II, and F, ranged from 1 to 6 relative units. Higher values and a much wider range were found for the large cells (4—25 relative units).

If the enzyme activities were expressed per unit dry mass the differences between the small and large cell types are largely eliminated. This is shown in

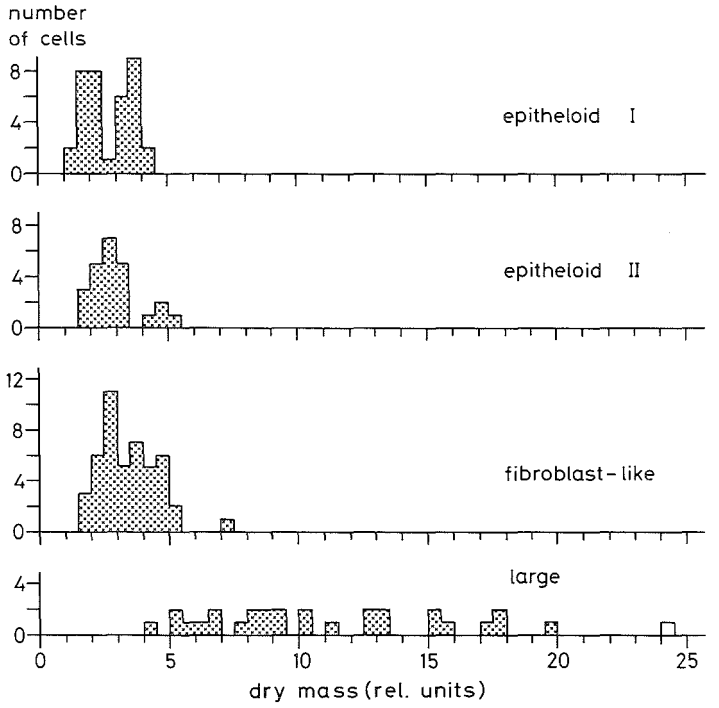

Fig. 2. Distribution of the dry mass per cell of different cell types in primary amniotic fluid cell cultures at 9 days of growth

Table 2 for the enzyme β-N-acetylglucosaminidase using the mean activities and dry mass values estimated in the separate experiments mentioned earlier. In additional experiments the β-N-acetylglucosaminidase activity and dry mass were measured in the same single cells. No significant difference of the hexosaminidase activities per unit of dry mass was found between the various amniotic fluid cell types.

Table 2. Combined microchemical β-N-acetylglucosaminidase assays and interferometric dry mass determination in different amniotic fluid cell types

Cell types	Theoretical[a]			Experimental		
	Mean act[b] per cell	Mean dry mass per cell	Mean act[c] per dry mass	Mean act[b] per cell	Dry mass per cell	Mean act[c] per dry mass
Epithelioid I	123	2.8	44.7	—	—	—
Epithelioid II	123	3.0	41.0	99	3.8	26 (22)
Fibroblast-like	107	3.4	31.3	94	3.7	24 (11)
Large	501	11.2	44.6	197	9.1	22 (7)

[a] The theoretical part originates from Figure 2 and Table 1
[b] Mean act per cell: moles 4 MU/h/cell $\times 10^{-14}$
[c] Mean act per dry mass: moles 4 MU/h/rel. unit dry mass $\times 10^{-14}$
Between ( ) the numbers of measured cells

Discussion

In primary amniotic fluid cultures, we have observed four distinct cell types. Following the morphologic description by Uhlendorf (1970), we have distinguished: the typical epithelioid (E I) and fibroblast-like (F) cell types, a type with morphologic and growth characteristics between E I and F (cf., Types E III in Sutherland et al., 1974, and AF in Hoehn et al., 1974), and one type of large, often multinuclear, cells (cf., Uhlendorf, 1970; Sutherland et al., 1974; Hoehn et al., 1974). Despite the strong heterogeneity with respect to morphology, size, number of nuclei, and growth characteristics, we have not further subdivided the large cells. In most primary amniotic fluid cultures, only a few large cells were observed within the colonies of other cell types or as slowly proliferating clones. High numbers of large cells were usually found in poorly growing cultures only, which supports the suggestion by Hoehn et al. (1974) that these large cells are senescent.

The present studies showed no significant difference in the activities of three lysosomal enzymes between the small cell Types E I, E II, and F, but much higher activities per cell were observed for the large cells. However, if the enzyme activities were expressed per unit of dry mass there is no significant difference between the small cell types and the large cells.

When prenatal diagnoses are based on biochemical assays of homogenates of subcultured amniotic fluid cells, no problems seem to be present concerning possible differences among various cell types (Melancon et al., 1971; Gerbie et al., 1972). However, if microchemical analyses are performed on small cell numbers isolated from primary amniotic fluid cell cultures (Galjaard et al., 1973, 1974; Kleijer et al., 1976; Niermeijer et al., 1975), it is important to establish the nature of the prevailing cell type(s). When rapid prenatal diagnoses are based on enzyme activities expressed per cell, the isolation of large cells should either be avoided or comparisons should be made with similar cells in primary cultures of control samples.

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paper II

Rapid Prenatal Diagnosis of GM_1-Gangliosidosis Using Microchemical Methods

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Summary. Prenatal diagnoses were established in 3 pregnancies at risk for GM_1-gangliosidosis at 9, 10, and 12 days after amniocentesis. β-galactosidase activities in cultured amniotic fluid cells were determined by microchemical assays in cell homogenates and in isolated groups of 10—30 freeze-dried cells. The latter method requires only a few hundred cells growing in one or more clones and will usually allow a diagnosis within 9—12 days after amniocentesis.

Introduction

Prenatal diagnoses have been made for about 30 inherited metabolic diseases (Burton et al., 1974; Hsu and Hirschhorn, 1974). The prenatal detection of GM_1-gangliosidosis has been described by Lowden et al. (1973), Kaback et al. (1973), and Booth et al. (1973).

Since cultured amniotic fluid cells are required for the biochemical assays, a period of 3—6 weeks has usually been necessary to establish a prenatal diagnosis for metabolic disorders. As amniocentesis is carried out in the 14th—16th week of pregnancy, it would be advantageous if the biochemical assays could be performed after about 2 weeks of cell cultivation, thus permitting a diagnosis before the mother experiences fetal movements.

Microchemical methods have been described which enable the assay of lysosomal enzyme activities on small numbers or even single cells (Galjaard et al., 1974a, b; Jongkind et al., 1974). Using these methods prenatal diagnoses could be established within 10—15 days after amniocentesis in pregnancies at risk for Pompe's disease (Galjaard et al., 1973; Niermeijer et al., 1975) and Fabry's disease (Galjaard et al., 1974c).

This report describes the prenatal monitoring of 3 pregnancies at risk for GM_1-gangliosidosis using microchemical methods. β-galactosidase activities were determined in isolated groups of 10—30 freeze-dried cultured amniotic fluid cells and the results were compared with those of assays on homogenates of larger numbers of cells and of enzyme assays on cell-free amniotic fluid.

Case Reports

Case I. The parents are of Turkish origin, living in W. Germany. Their son showed the clinical characteristics of GM_1-gangliosidosis and a deficient activity of β-galactosidase in cultured skin fibroblasts (Results, Table 2). Amniocentesis was performed in Germany in the 16th week of pregnancy but, because of a failure during transport of the sample, no cell culture could be established. A second amniocentesis was performed in the 19th week and a satisfactory cell growth was obtained.

Case II. The parents are nonconsanguinous and of Dutch origin. Their daughter showed the clinical characteristics of a lipidosis and died at the age of 18 months. Investigations of a liver biopsy and of liver and brain tissue obtained at autopsy revealed extensive vacuolization and an accumulation of sphingolipids in the liver. β-galactosidase activity was almost completeley absent in homogenates of liver and moderately decreased in brain (Dr. G. J. M. Hooghwinkel, Leiden). Fibroblasts of the patient were reported to show a β-galactosidase deficiency, but they were not available at the time of amniocentesis (16th week of pregnancy).

Case III. The parents are consanguinous and of Dutch origin. Two of their children died with the symptoms of a progressive psychomotor retardation at the age of 15 and 18 months, respectively. Lymphocytes of both children showed vacuolization, and in a liver biopsy of the second child an accumulation of glycolipids (Dr. G. J. M. Hooghwinkel, Leiden) and a deficiency of β-galactosidase (Prof. F. van Hoof, Louvain) were found. Cultured fibroblasts of the patients were not available. Amniocentesis was performed in the 17th week of pregnancy.

Materials and Methods

Cultivation and Preparation of Cell Material. Amniotic fluid cells and skin fibroblasts were cultured as described earlier (Niermeijer et al., 1975).

Primary cultures of amniotic fluid cells from the pregnancies at risk for GM_1-gangliosidosis and from controls (obtained from pregnancies at risk for neural tube defects or chromosomal disorders) were grown on 35 mm Falcon dishes if microchemical assays were to be performed on cell homogenates. Fibroblast strains (6th—12th passage) from patients with GM_1-gangliosidosis, heterozygous parents, and controls were grown in glass bottles and harvested 6—10 days after the last subculture. Cell homogenates were prepared in saline by sonication of suspensions at a concentration of 2×10^6 cells/ml.

For microchemical enzyme assays on small groups of freeze-dried cells, primary cultures of amniotic fluid cells were grown on dishes with a thin plastic bottom. When sufficient clones were available the dishes were freeze-dried and 10—20 groups, each consisting of 10—30 cells were isolated according to methods described by Galjaard et al. (1974a, b). Similar preparations were made of fibroblast cultures 4 days after seeding 2×10^4 cells per dish.

Cell-free amniotic fluid samples were stored at —70° C for several days to weeks before use.

Microchemical Enzyme Assays. Enzyme assays on cell homogenates were performed by incubation of 1 µl homogenate with 2 µl of the appropriate methylumbelliferone substrate solutions and measurement of the fluorescence in a final volume of 500 µl sodium carbonate buffer (pH 10.7). Pieces of plastic foil containing 10—30 counted freeze-dried cells were incubated for 1 h in 0.4 µl substrate solution and, after addition of 5 µl sodium carbonate buffer, the fluorescence was measured in capillaries, using a Leitz MPV microspectrofluorometer. Enzyme assays in amniotic fluid supernatants were performed by incubation of 1 µl of 10 × diluted amniotic fluids with 2 µl of the appropriate substrate for β-N-acetylglucosaminidase or 50 µl undiluted amniotic fluid with 100 µl of the appropriate substrate for β-galactosidase. Details of the procedures used have been described elsewhere for the assay of β-galactosidase (Galjaard et al., 1974b), β-N-acetylglucosaminidase (Galjaard et al., 1974d), and α-glucosidase (Galjaard et al., 1973).

The protein content of the cell homogenates and amniotic fluid supernatants was measured according to Lowry et al. (1951) using a final volume of 60 µl.

Results

a) Microchemical Assays on Isolated Groups of Freeze-Dried Cells. Amniotic fluid cells from case I could be isolated after 9 days of growth from several colonies consisting of small cells of intermediate type (i.e., morphologically between the

Table 1. β-galactosidase activities in isolated freeze-dried cells

Cell type	β-galactosidase activity[a]		
	case I	case II	case III
Amniotic fluid cells			
Pregnancy at risk			
small cells	16.1 (17)	5.8 (14)	2.9 (23)
large cells		—	93.8 (9)
Control pregnancy			
small cells	30.3 (9)	11.7 (7)	3.6 (14)
large cells		63.8 (9)	130.4 (5)
Fibroblasts			
control	25.5 (15)	34.3 (19)	—
GM_1 patient (unrelated)	0.0 (20)	0.5 (12)	—

[a] The mean β-galactosidase activities (10^{-14} moles/h/cell) were calculated from the activities in (n) cell groups, each containing 10—30 cells; the standard errors of the mean were 5—10% for the fibroblasts and 10—15% and 13—22%, respectively, for small and large types of amniotic fluid cells.

epithelial and fibroblastlike types) and large, sometimes multinuclear, cells in various proportions. From case II only one colony with cells of intermediate type was obtained after 12 days of growth. Ten days after initiation of the culture in case III, homogeneous groups of small (epithelial, intermediate, and fibroblast-like) and large multinuclear amniotic fluid cells were isolated. Comparable cell groups were isolated from the age-matched control amniotic fluid cultures, i.e., groups containing small and large cells from the control in case I and separate groups of either small or large cells from the controls of cases II and III.

Table 1 shows the mean β-galactosidase activities in the groups of freeze-dried cells isolated from the cultures of amniotic fluid cells from the pregnancies at risk and control pregnancies and of fibroblasts from normal individuals or an unrelated patient with GM_1-gangliosidosis, type 2.

The large cells in case II (control culture) and case III (control and diagnostic cultures) exhibited much higher β-galactosidase activities per cell than the smaller cell types. The ranges of activities in the groups of small cell types and of the large type were quite distinct, e.g., in the control of case II: 7.6—17.6 and 41—116 $\times$ 10^{-14} moles/h/cell, respectively. The activities in the cell groups of case I and its control were higher than those in the small cell types (e.g., case III) and probably reflect the presence of large cells, which were observed but not separated from the smaller cells.

The mean β-galactosidase activities in the cells of the 3 cases at risk varied from 50 to 90% of the control activities if either all cell groups (case I) or groups of similar cell type were compared (cases II and III). In each of the 3 pregnancies at risk, however, all cell types showed a clear β-galactosidase activity, indicating that the fetus was not affected.

Table 2. β-Galactosidase activities in cell homogenates

A. Prenatal diagnoses

Cell origin	Case I		Case II		Case III	
	β-gal.[a]	hex.[a]	β-gal.	α-glu.[a]	β-gal.	hex.
Amniotic fluid cells						
controls[b]	615	4600	580	18	1109	15200
pregnancy at risk	690	5400	444	40	1000	16800
Fibroblasts						
control	780	11300	1518	65	1260	14100
heterozygotes[c]	—	—	655	84	—	—
GM_1 patient[d]	63	12500	66	43	—	—

B. Enzyme activities in independent control cultures[e]

Enzymes	Fibroblasts			Amniotic fluid cells		
	$\bar{X}$	(n)	S.D.	$\bar{X}$	(n)	S.D.
β-galactosidase	815	(29)	191	856	(28)	356
β-hexosaminidase	12500	(18)	5461	9931	(18)	4507
α-glucosidase	85	(13)	36	29	(32)	15

[a] Activities of β-galactosidase and the reference enzymes β-N-acetyl-glucosaminidase and α-glucosidase: nmoles/h/mg protein.

[b] 2 and 3 controls were used in case I and II, respectively.

[c] Parents in case II.

[d] Index patient in case I; an unrelated patient in case II.

[e] Mean enzyme levels ($\bar{X}$) and standard deviations (S.D.) were obtained from (n) independent assays of the 3 enzymes in 15, 11 and 7 different control fibroblast strains and 24, 16, and 22 different amniotic fluid cultures, respectively.

b) Cell Homogenate Studies. Homogenates of the amniotic fluid cells from cases I, II, and III were prepared and analyzed after a culturing period of 12, 20, and 15 days, respectively. The GM_1-gangliosidosis fibroblasts originated from the affected child in family I or from an unrelated patient with GM_1-gangliosidosis type 2 in case II.

Table 2, A shows the specific activities of β-galactosidase for all cultures and the activities of β-N-acetylglucosaminidase (cases I and III) or α-glucosidase (case II) measured as reference lysosomal enzymes. For comparison the mean specific activities and standard deviations of the three enzymes, obtained from a larger number of independent assays in control fibroblast strains and amniotic fluid cultures, are presented in Table 2, B.

The β-galactosidase activities in the amniotic fluid cultures of all 3 pregnancies, at risk were in the normal range, which indicated that the fetuses were not affected.

c) Studies of β-Galactosidase in Cell-Free Amniotic Fluid. β-galactosidase and β-N-acetylglucosaminidase activities were determined in amniotic fluid supernatants from cases I, II, and III and from 13 control pregnancies. All fluids were received within a few hours after amniocentesis, except those of case I, which had been in transport at room temperature for 24 h.

Table 3. β-galactosidase activities in cell-free amniotic fluid

Amniotic fluid	Week of pregnancy	Protein (mg/ml)	β-galactosidase (nmoles/h/ml)	Hexosaminidase (nmoles/h/ml)
Controls[a]	14—16	5.0	2.33	2192
(13 samples)		(3.4—6.4)	(0.81—4.24)	(1323—2792)
Case I				
1st sample	16	4.8	0.46	2169
2nd sample	19	5.5	0.16	1518
Case II	16	7.7	1.87	2127
Case III	17	4.4	2.83	1494

[a] Mean and range of protein content and enzyme activities.

As shown in Table 3, the β-galactosidase activity in the amniotic fluid supernatants of cases II and III was in the normal range; in contrast the β-galactosidase levels were low in both samples of case I despite normal levels for hexosaminidase. The stability of β-galactosidase in amniotic fluid at room temperature was investigated to check whether the longer period of transport of the amniotic fluid of case I could have caused the low activities. Five amniotic fluid samples were stored at room temperature for 1, 2, 3, and 5 days, but showed only a slight decline (10%) of β-galactosidase activity.

Discussion

In each of the 3 pregnancies at risk for GM_1-gangliosidosis, a nonaffected child was predicted by the results of β-galactosidase assays in the cultured amniotic fluid cells. The diagnosis was confirmed in case II by the demonstration of a normal β-galactosidase activity in the leukocytes of the child after birth (Dr. J. F. Koster, Rotterdam). The baby born in case I was reported to have no clinical abnormalities until about 1 year of age, but unfortunately no cell material became available to us; the pregnancy in case III has not yet come to term.

The period needed to establish a prenatal diagnosis for GM_1-gangliosidosis, which has varied from 2½ (Lowden et al., 1973) to 4 weeks (Kaback et al., 1973; Booth et al., 1973), could be reduced to 12, 20, and 15 days in the present cases by using microliter volumes of cell homogenates for the microchemical assays of enzyme activities and protein content. A further reduction to 9, 12, and 10 days could be achieved by using micromethods in which small numbers of cells (1—100) are counted and analyzed, thus avoiding the need of a protein assay (Galjaard et al., 1974a, b).

As most amniotic fluid cell cultures will show outgrowth of cell colonies of different morphology (Hoehn et al., 1974) attention was paid in this study to the possible different enzyme activities in such cell types. It was shown that the β-galactosidase activity, expressed per cell, in the large (often multinuclear) cells is usually much higher and also more variable than in the smaller cell types (e.g., case III, Table 1). Single cell dry mass measurements and more detailed studies of the activities of several lysosomal enzymes in epithelial, intermediate, fibroblast-like, and large cells have been performed and will be reported elsewhere (E. Van der Veer et al.). These studies indicated that an early prenatal diagnosis should be based on a separate analysis of small and large cells; alternatively, the isolation of large cells could be avoided.

Other approaches applied for rapid prenatal diagnosis are a direct assay of β-galactosidase in cell-free amniotic fluid and cytochemical staining for β-galactosidase in cultured cells. As shown in Table 3 β-galactosidase is clearly detectable in amniotic fluid but the levels reported by different groups differ by orders of magnitude (Butterworth et al., 1974; Lowden et al., 1973; Huijing et al., 1973). A deficiency of β-galactosidase in amniotic fluid has been reported in a pregnancy in which an affected fetus was shown (Lowden et al., 1973). In one of the pregnancies at risk in this study (case I) a remarkably low, though not deficient, β-galactosidase activity was observed in two different amniotic fluid supernatants, whereas a normal activity was found in the cultured amniotic fluid cells. It is unlikely that the enzyme activity was lost during transport, since it was shown that β-galactosidase is sufficiently stable in amniotic fluid during storage of up to 5 days at room temperature. Clearly more data are required on β-galactosidase levels in amniotic fluid in normal and affected cases, before this approach can be of any value for diagnostic purposes.

The cytochemical staining procedure for β-galactosidase as applied in prenatal diagnosis by Kaback et al. (1973) and ourselves may provide qualitative information within 7—10 days after amniocentesis but, in our opinion, such results should always be confirmed by quantitative methods.

Quantitative microtechniques for enzyme assays in either isolated freeze-dried cells or small volumes of cell homogenates enable a reduction of the period between amniocentesis and prenatal diagnosis to 10—20 days for several metabolic disorders (Galjaard et al., 1973, 1974c; Niermeijer et al., 1975; Kleijer et al., 1975).

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paper III

EXPRESSION OF LYSOSOMAL ENZYMES IN HUMAN MUTANT FIBROBLAST-CHICK ERYTHROCYTE HETEROKARYONS

E. Van der Veer, R.A. Barneveld and A.J.J. Reuser

SUMMARY

The generation of enzymes located in lysosomes, in cytosol or in endoplasmatic reticulum/Golgi complex is studied in heterokaryons in which chick erythrocyte nuclei are reactivated. The lysosomal enzymes, α-glucosidase (α-glu) and β-galactosidase (β-gal) are synthesized in heterokaryons obtained after fusion of chick erythrocytes with human fibroblasts of patients with Pompe's disease (α-glu deficient) and G_{M1}-gangliosidosis (β-gal deficient), respectively. The enzymes appear to be of chick origin and their activities can be detected at first around 4 days after fusion i.e. at a time when the nucleoli in the erythrocyte nuclei have been reactivated. Maximal activities are reached around 15 days after fusion. No generation of the lysosomal enzyme β-hexosaminidase is detected in the heterokaryons up to 23 days after fusion of chick erythrocyte with either β-hexosaminidase A and B deficient fibroblasts (Sandhoff's disease) or β-hexosaminidase A deficient fibroblasts (Tay-Sachs disease). Similarly no expression of the cytosol enzyme glucose-6-phosphate dehydrogenase (G6PD) is found up to 30 days after fusion, when chick erythrocytes are fused with fibroblasts from two different G6PD deficient cell strains (residual activities of 4% and 20%, respectively). Indirectly we examined N-acetyl-glucosamine-1-phosphate transferase activity, an enzyme located in the endoplasmic reticulum/Golgi region. This enzyme is needed for the phosphorylation of the lysosomal hydrolases and absence of its activity is the cause of the multiple lysosomal enzyme deficiencies in patients with I-cell disease. The retention of both, chick and human β-galactosidase in the experiments in which I-cell fibroblasts were fused with chick erythrocytes indicate a reactivation of the gene coding for this phosphorylating enzyme. It also implies that this step in the processing of human lysosomal enzymes is not species specific.

Exp.Cell Res., accepted for publication

INTRODUCTION

Various chick characteristics are expressed in heterokaryons containing reactivated chick erythrocyte nuclei (for review see 1.2). With the reappearance of the chick nucleolus heterokaryons produce chick surface antigens (3) chick specific nucleolar antigens (4) and chick surface receptors (5). Chick ribosomal RNA is found in the cytoplasm of the heterokaryons (6). Several enzymes involved in nucleic acid metabolism are detectable within the first 24 hours after fusion: hypoxanthine guanine phosphoribosyl transferase (7) adenosine phosphoribosyl transferase (8) and cytoplasmic thymidine kinase (9). Restoration of repair DNA synthesis is found in human fibroblasts of patients with xeroderma pigmentosum after fusion with chick erythrocytes (10). This implies that enzymes involved in repair DNA synthesis are also expressed upon reactivation of the chick nucleus. All these markers are examples of reactivation of constitutive functions. The only example of the reactivation of a differentiation function has been published by Linder et al.(11). They demonstrated the expression of globin polypeptides translated from chick mRNA in the chicken erythrocyte-rat L6 myoblast heterokaryons.

Although a new polarization of the cytoplasmic organelles and the development of a Golgi apparatus in the vicinity of the chick nucleus is observed (12), little is known about the expression of markers residing in the cytoplasmic organelles. Lysosomal enzymes are interesting in this respect because of the absence of a lysosomal apparatus of chick origin in the heterokaryons immediately after fusion. In this report the induction of these enzymes is studied and the species specificity of an early step in their post-translational modifications is investigated. Recent work has shown that lysosomal enzymes are synthesized as large precursor molecules which are modified during their receptor mediated intracellular transport into the lysosomes (for review see 13). Three enzymes known to undergo such processing, β-galactosidase (β-gal) (14), β-hexosaminidase (β-hex) and α-glucosidase (α-glu) (15) were investigated in the present report. We examined indirectly N-acetyl glucosamine-1-phosphate transferase activity, which is located in the ER/Golgi region. In I-cell disease this enzyme is the primary defect, which is responsible for the multiple lysosomal

deficiencies (16,17). Finally we investigated a constitutive cytosolic enzyme glucose-6-phosphate dehydrogenase (G6PD).

MATERIALS AND METHODS

Cells

The primary human fibroblast strains used in these studies are derived from patients with G_{M1}-gangliosidosis (GAL^-); Sandhoff's disease ($HEX\ A^-B^-$); Tay-Sachs disease ($HEX\ A^-$); Pompe's disease (GLU^-); I-cell disease (I-cell); glucose-6-phosphate dehydrogenase deficiency with either 4% or 20% residual activity ($G6PD^-$); and control human fibroblasts (C5RO). The cells were grown in Ham's F10 medium supplemented with 4% foetal and 4% newborn calf serum and antibiotics. Prior to fusion chick erythrocytes were obtained by collecting blood from the allantoic vessels of 11-13 days old embryos.

Cell fusion

Heterokaryons were prepared by fusing embryonic chick erythrocytes with human fibroblasts, using β-propiolactone inactivated Sendai virus (200-300 HAU/ml) using a modified method from Harris (for details see 10). Within the first 24 hours after fusion the cultures were washed extensively to remove the non-attached erythrocytes. The fusion index at each experimental time point was determined by counting 500 - 1000 nuclei in stained preparations. In the experiments we have used as lowest level either 15% heterokaryons (i.e. nuclei in heterokaryons expressed as % of total nuclei scored) or 10% erythrocyte nuclei (i.e. nuclei of chick erythrocytes in heterokaryons expressed as percentage of total nuclei scored). To extend the lifespan of the heterokaryons the DNA replication and mitosis was suppressed by treating the human parental cells with 0.2 µg/ml mitomycin C for 16 hr (11). After washing twice with saline, the cells were kept in normal medium for a few hours before starting the fusion procedure.

Measurement of enzyme activities in cell homogenates

For enzyme assays the cultures after fusion were harvested by trypsinization, washed with saline and homogenized by repeated freez-

ing and thawing. For quantitative lysosomal enzyme assays 10 µl of cell homogenates was incubated with 20 µl of appropriate 4-methylumbelliferyl glycoside substrate (4-MU-substrate) for 30 min. at 37°C. The assay conditions were as described for β-gal (18), β-hex (19) and α-glu (20). G6PD activity was measured according to Jongkind et al. (21). The protein content of the cell homogenates was measured according to Lowry et al. (22).

Microchemical β-hexosaminidase assay

For the microchemical enzyme assay the cultures after fusion were grown on dishes with a thin plastic bottom and freeze-dried. Individual cells were isolated, as described earlier (23). The pieces of plastic foil with one cell attached were incubated under hexadecane-paraffin oil (40/60, v/v) for 1 hour in 0.09 µl of 4-MU-substrate. After addition of 1.0 µl sodium carbonate buffer pH 10.7 the fluorescence was measured with an inverted microscope equipped with a vertical illuminator for incident light (24).

Electrophoresis

Electrophoresis of β-galactosidase was performed on Cellulose acetate gels (Cellogel, Chemotron, Milan, Italy) in a 0.05 M sodium-β-glycerophosphate buffer pH 6.2 at an initial voltage of 200 Volts keeping the amperage constant for 3 hours at 4°C (25).

Immunoprecipitation

For immunological studies an antiserum was used that was raised in rabbits against purified human placenta β-galactosidase, α-glucosidase and β-hexosaminidase (The latter two are generous gifts of Dr. J.M. Tager, Lab. of Biochemistry, University of Amsterdam, and Dr. J. Stirling, Queen Elizabeth College, University of London, respectively). An aliquot of 15 µl cell homogenate was incubated overnight at 4°C with an equal volume of serially diluted antiserum. Immmunocomplexes were spun down at 35.000 g for 30 minutes. The activity of unprecipitated enzyme was measured in the supernatant using appropriate 4-MU-substrate.

Secretion and uptake of chick hydrolases

Chick fibroblasts in passages 3-5 were grown to confluency in medium with 5% inactivated foetal calf serum, after which the cells were kept in serum free medium supplemented with 10 mM NH_4Cl for 48 hrs, to stimulate the secretion of chick hydrolases (16,26). The medium was concentrated (Amicon PM 10 filter) and dialyzed against fresh serum free medium. The final concentration of NH_4Cl was 0.1 mM. This preparation which contained β-hex, 296 nmoles 4-MU/hr/ml and β-gal, 47 nmoles 4-MU/hr/ml was supplemented with 2% inactivated foetal calf serum before adding to the mutant fibroblasts. After 24 hours the medium was replaced by fresh medium with 5% inactivated foetal calf serum and the cells were harvested by trypsinization at 0,1,2,3 and 6 days after medium change. The enzyme activities were measured as described above.

RESULTS

Expression of β-hexosaminidase

Fibroblasts of a patient with Sandhoff's disease (HEX A^-B^-) which are deficient in β-hexosaminidase A and B (β-hex) were fused with chick erythrocytes and at different time intervals after fusion the β-hex activities were measured in homogenates. As a control, β-hex deficient cells were fused in the absence of chick erythrocytes (HEX A^-B^- - HEX A^-B^-). The results are presented in Table 1. The β-hex activity, remained at the level observed in human homokaryons for a period of 23 days after fusion. Morphologically the erythrocyte nuclei showed a normal reactivation pattern in these heterokaryons.

By using the microchemical assay for β-hex the enzyme activity was measured in single heterokaryons that had different ratio's of human and chick nuclei. The β-hex activities in heterokaryons with either one, two or three and more erythrocyte nuclei at 8 days after fusion were no greater than the activity in the β-hex deficient homokaryons (Fig. 1).

Similarly, fibroblasts of a patient with Tay-Sachs disease (HEX A^-), which are selectively deficient in β-hexosaminidase A, were fused with chick erythrocytes. Since β-hexosaminidase B activity of these human

Table 1.

HEXOSAMINIDASE ASSAYS ON CELL HOMOGENATES AFTER FUSION OF CHICK ERYTHROCYTES AND FIBROBLASTS FROM A PATIENT WITH SANDHOFF'S DISEASE

Days After Fusion	HEX A^-B^- x ERY			HEX A^-B^- x HEX A^-B^-
	% HET.K[2]	% ERY[3]	β-hex act[1]	β-hex act[1]
2	65	38	107	108
4	49	27	142	130
8	45	25	83	130
16	31	15	86	74
23	35	17	98	112

Control Cultures	β-hex act[1]
Human Fibroblasts	5612
Chick Fibroblasts	904
Chick Red Blood Cells	52

1) β-hex activities in nmoles 4-MU/hr/mg protein

2) % HET.K = nuclei in heterokaryons expressed as percentage of total nuclei scored; ± 1000 nuclei were counted

3) % ERY = nuclei of chick erythrocytes in heterokaryons expressed as percentage of total nuclei scored.

mutant fibroblasts would obscure any expression of chick hexosaminidase in the heterokaryons, immunological methods had to be used. The immunological precipitation patterns of β-hexosaminidase in the human-chick heterokaryons are compared with the patterns of the human homokaryons by immunotitration with anti human β-hex antiserum. The antiserum cross reacts with both A and B forms of β-hex and does not cross react with chick β-hexosaminidase (Fig.2). The precipitation patterns of the Tay-Sachs fibroblast-chick erythrocyte heterokaryons harvested at 2, 8 and 15 days after fusion resemble the patterns of the Tay-Sachs homokaryons, and do not show any extra chick β-hex components. A limitation of this method is that if a heteropolymer between chick and human subunits is formed, it would not be distinguishable but would be precipitated in the same fashion as homologous human enzyme components.

In order to exclude the possibility that chick β-hex is expressed, but is rapidly degraded in the human lysosomal environment, we studied the stability of chick β-hex that had been taken up by human mutant fibroblasts. The enzymes used were chick hydrolases, which had been secreted into the medium by chick fibroblasts grown in the presence of 10 mM NH_4Cl (16,26). After a 24 hours uptake of these chick hydrolases the human mutant fibroblasts were harvested at 0,1,2,3 and 6 days (for details see Material and Methods) and the activity of the chick hydrolases was measured in the mutant fibroblasts. During these 6 days no significant decrease of chick β-hexosaminidase and of β-galactosidase was seen (Table 2).

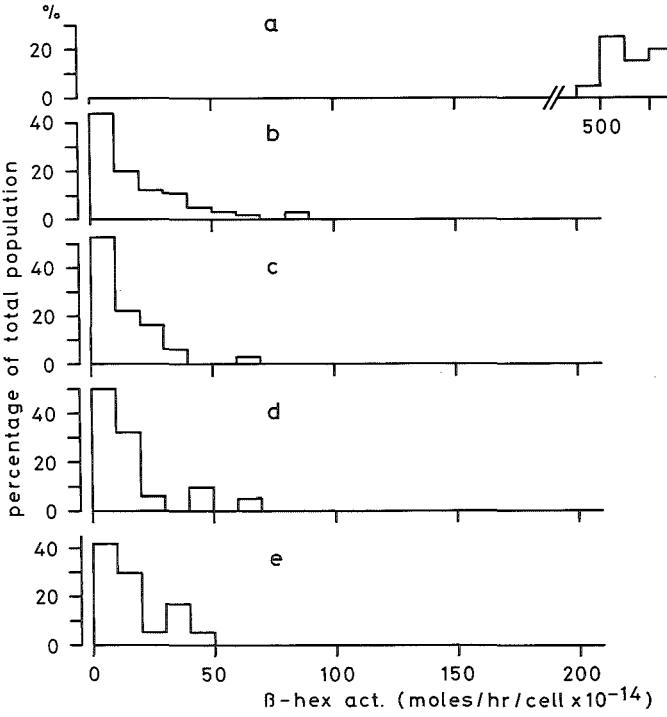

Fig. 1 β-Hexosaminidase activities in single heterokaryons obtained after fusion of HEX A^-B^- fibroblasts with chick erythrocytes. a) C5RO (n=20); b) HEX A^-B^- x HEX A^-B^- (n=64); the HEX A^-B^- x chick erythrocytes fusion; c) heterokaryons with 1 human and 1 chick nucleus (n=32); d) heterokaryons with 1 human and 2 chick nuclei (n=22); e) heterokaryons with 1 human and 3 or more chick nuclei (n=24).

Fig. 2

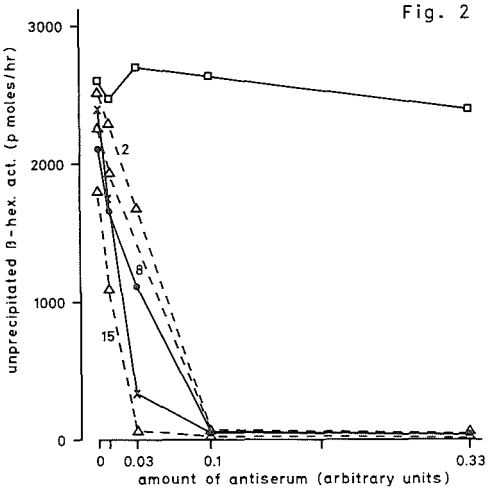

Fig. 3

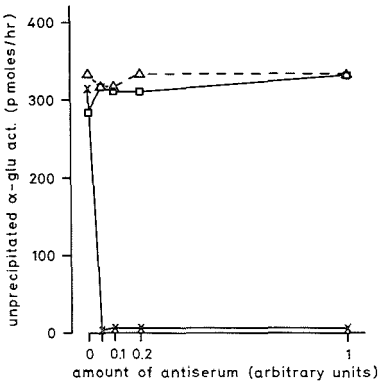

Fig. 2 Immunoprecipitation of β-hexosaminidase in homogenates of β-hexosaminidase A deficient fibroblasts fused with chick erythrocytes, 2, 8 and 15 days after fusion by an antiserum raised against human placenta β-hexosaminidase □—□ chick fibroblasts; X——X C5RO; ●——● HEX A^- x HEX A^-; △-----△ HEX A^- x chick erythrocyte fusion: 2 days after fusion, 95% HET.K., 78% ERY; 8 days after fusion, 88% HET.K., 63% ERY; 15 days after fusion, 84% HET.K., 55% ERY.

Fig. 3 Immunoprecipitation of α-glucosidase in an homogenate of GLU^- fibroblasts fused with chick erythrocytes, 15 days after fusion, by an antiserum raised against human placenta α-glucosidase □—□ chick fibroblasts; X——X C5RO; △---△ GLU^- x chick erythrocytes, 61% HET.K., 32% ERY.

Expression of α-glucosidase

In cultured fibroblasts of the progressive infantile form of Pompe's disease the activity of acid α-glucosidase (α-glu) is around 1% of normal human level. Starting at about 4 days after fusion of Pompe's fibroblasts with chick erythrocytes α-glu activity can be detected in homogenates of the cultures. A maximum activity of 45 nmoles 4-MU/hr/mg protein is reached around 15 days after fusion, at which time 61% of the nuclei are still within heterokaryons (Table 3). This α-glu activity is 60 times the activity measured in homogenates of cultures

Table 2.

STABILITY OF CHICK FIBROBLAST HYDROLASES INGESTED BY MUTANT FIBROBLASTS

Hours after addition	HEX A^-B^- β-hex act[1)]	GAL^- β-gal act[1)]
0	1247	30
24	1100	30
48	1309	36
72	1370	28
144	1010	22
No addition	89	1

Control Cultures	β-hex act	β-gal act
Human Fibroblasts	7897	571
Chick Fibroblasts	795	196

1) Hydrolase activities in nmoles 4-MU/hr/mg protein

after fusion of Pompe cells only, and lies between the specific activities found in normal human and chick fibroblasts (52.6 and 32.4 nmoles 4-MU/hr/mg protein respectively). At 23 days after fusion the percentage of heterokaryons in the cultures is decreased markedly (21%) but the α-glu activity is still 20x higher than that in the control fusion (Pompe - Pompe fibroblasts). The immunological characteristics of the control human and chick fibroblast α-glu and of the α-glu in the Pompe fibroblast-chick erythrocyte homogenate, harvested 15 days after fusion (see Table 3) as compared by immunotitration with anti human α-glucosidase antiserum are shown in Fig.3. Under the circumstances described in Materials and Methods α-glu from control human fibroblasts is completely precipitated. The antiserum does not cross-react with chick α-glu. The α-glu found in the human-chick fusion fails to react with the human specific α-glucosidase antiserum, indicating that the activated α-glucosidase is of chick origin.

Table 3.

α-GLUCOSIDASE ASSAYS ON CELL HOMOGENATES AFTER FUSION OF CHICK ERYTHROCYTES AND FIBROBLASTS FROM A PATIENT WITH POMPE'S DISEASE

Days After Fusion	GLU^- x ERY			GLU^- x GLU^-
	% HET.K[2]	% ERY[3]	α-glu act[1]	α-glu act.
2	73	44	1.1	0.7
4	73	44	2.0	1.1
8	73	42	12.9	0.7
15	61	32	45.0	0.4
23	21	10	17.6	0.9

Control Cultures	α-glu act
Human Fibroblasts	52.6
Chick Fibroblasts	32.4
Chick Red Blood Cells	1.0

1) α-glu activities in nmoles 4-MU/hr/mg protein

2) and 3) see Table 1

Expression of β-galactosidase

β-gal activity in a cell strain of a patient with the infantile form of G_{M1}-gangliosidosis is less than 1% of the control values. At 8 days after fusion the β-gal activity is 20.2 nmoles 4-MU/hr/mg protein in the human-chick fusion i.e. 9 times the activity in the corresponding human-human fusion (Table 4). Concomitant with the decrease of the fusion index a decrease in β-gal activity is seen from 8 days to 30 days after fusion. To compare several experiments with different fusion indices, the ratio of β-gal activity to fusion index was plotted against time after fusion (Fig.4). For the first two days no β-gal activity is seen; at 4 days an increase in β-gal activity is detect-

Table 4.

β-GALACTOSIDASE ASSAYS ON CELL HOMOGENATES AFTER FUSION OF CHICK ERYTHROCYTES AND FIBROBLASTS FROM A PATIENT WITH GM_1-GANGLIOSIDOSIS

Days After Fusion	GAL^- x ERY			GAL^- x GAL^-
	% HET.K[2]	% ERY[3]	β-gal act[1]	β-gal act
8	86	52	20.2	2.2
15	70	37	14.9	2.9
23	43	22	9.4	2.6
30	38	17	6.1	2.4

Control Cultures	β-gal act
Human Fibroblasts	507
Chick Fibroblasts	183
Chick Red Blood Cells	6.4

1) β-gal activities in nmoles 4-MU/hr/mg protein
for 2) and 3) see Table 1.

able which reaches a maximum at 15 days after fusion from which point the activity decreases again, probably as a result of diminished viability of the heterokaryons.

The immunological characteristics of β-gal of control human and chick fibroblasts and of the human-chick fusion are compared by immunotitration with an antiserum raised against human placental β-galactosidase (Fig.5). The control human fibroblast β-galactosidase is completely precipitated and the antiserum does not cross-react with chick β-galactosidase. Most of the β-gal found in human-chick homogenate, at 8 days after fusion, fails to react with the human specific β-galactosidase antiserum. The small amount of β-gal activity which does react may represent the residual activity expected in the human GAL^- strain.

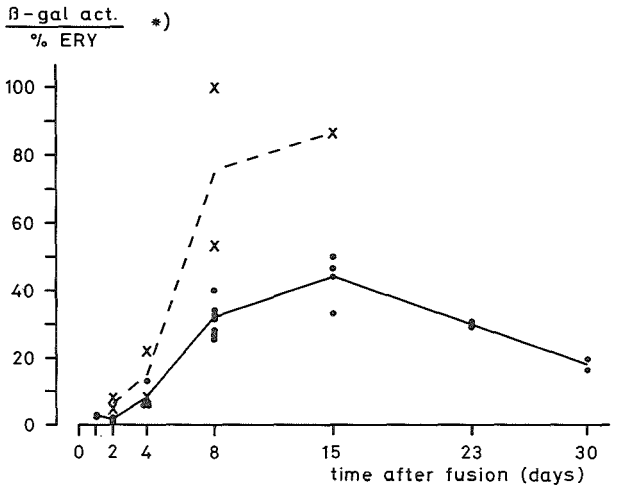

Fig. 4 Fusion studies between chick erythrocytes and I-cells or G_{M1}-gangliosidosis fibroblasts. Time dependent relationship between ß-galactosidase expression and fusion index. ●——— GAL^- x ERY. X------ I-cell x ERY. x) see Table 1 and 4.

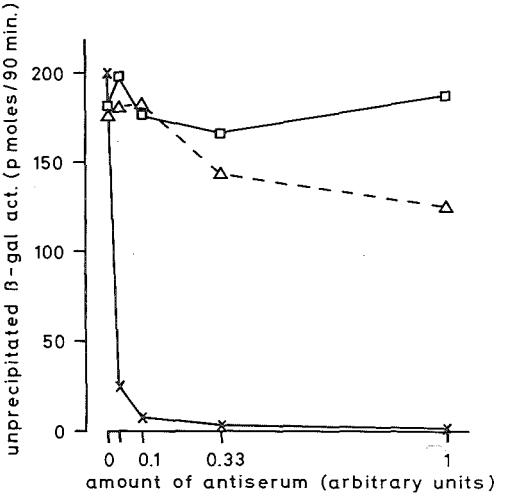

Fig. 5 Immunoprecipitation of ß-galactosidase in an homogenate of GAL^- fibroblasts fused with chick erythrocytes, 8 days after fusion by an antiserum raised against human placenta ß-galactosidase □——□ chick fibroblasts; X——X C5RO △---△ GAL^- x chick erythrocytes, 86% HET.K., 52% ERY.

The electrophoretic patterns (Fig. 6a) are consistent with these findings. The band of activity of human-chick lysate, at 8 days after fusion (lane 2) is at the same position as the control chick fibroblast band (lane 4). The weak chick erythrocyte band (lane 3) may be ascribed to the presence of granulocytes in this preparation.
No band of activity is detected in the human-human lysate although comparable amounts of protein are used in lane 1 and lane 2. Thus it seems very likely that the reactivated β-galactosidase is of chick origin.

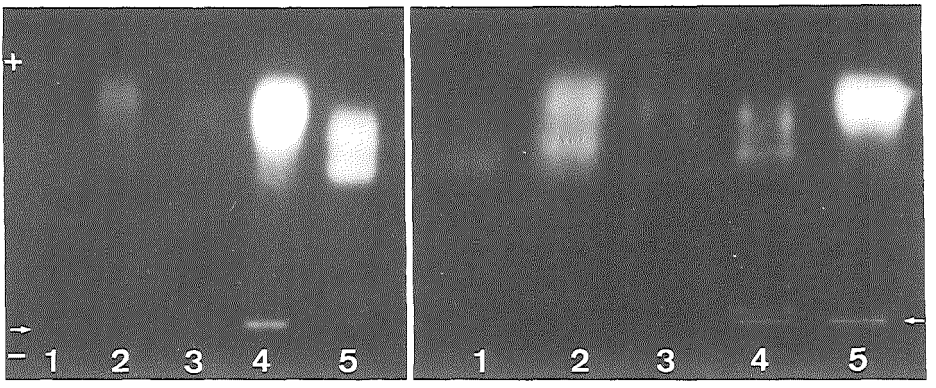

Fig. 6 Electrophoretic pattern of β-galactosidase of the GAL^- x chick erythrocyte (a) and I-cell x chick erythrocyte (b) fusions; a) lane 1 GAL^- x GAL^- (33 µg protein); lane 2 GAL^- x chick erythrocytes (25 µg); lane 3 chick erythrocytes (93 µg); lane 4 chick fibroblasts (2.3 µg); lane 5 control human fibroblasts (1.6 µg). b) lane 1 I-cell x I-cell (79 µg protein); lane 2 I-cell x chick erythrocytes (84 µg); lane 3 chick erythrocytes (185 µg); lane 4 control human fibroblasts (2.5 µgr); lane 5 chick fibroblasts (18 µg).

Apparent expression of N-acetyl glucosamine-1-phosphate transferase

The recent discovery by Hasilik et al. (16) and by Reitman et al. (17) that the defect in I-cell disease is due to a genetic deficiency of N-acetyl glucosamine-1-phosphate transferase allows us to study the reactivation of this endoplasmatic reticulum/Golgi complex (ER/Golgi) enzyme in I-cell fibroblast-chick erythrocyte heterokaryons.

Table 5.

β-GALACTOSIDASE ASSAYS ON CELL HOMOGENATES AFTER FUSION OF CHICK ERYTHROCYTE AND FIBROBLASTS FROM A PATIENT WITH I-CELL DISEASE

Days After Fusion	I-Cell x ERY			I-Cell x I-Cell
	% HET.K[2]	% ERY[3]	β-Gal act[1]	β-gal act[1]
2	53	32	7.7	5.5
4	55	33	7.7	5.4
8	42	24	17.5	5.0
15	31	17	19.5	4.5

Control Cultures	β-gal act[1]
Human Fibroblasts	507
Chick Fibroblasts	183
Chick Red Blood Cells	6.4

For 1), 2) and 3) see Table 4 and 1

The expression of this particular enzyme was studied indirectly by measuring the activity of human and chick β-galactosidase in the heterokaryons. Before fusion, β-gal is hardly present in I-cell fibroblasts because its precursor form is secreted into the medium as a result of deficient phosphorylation of mannose residues on the enzyme precursors. In the I-cell-chick fusion the β-gal activity is increased at 8 and 15 days after fusion compared with the I-cell homokaryon fusion (Table 5). Comparing the level of β-gal activity in these heterokaryons and that obtained by fusion of erythrocytes with β-gal deficient fibroblasts (GAL^-) a much higher level of β-gal activity was detected in the former case (Fig.4).

To test whether this difference is due to the retention of the human enzyme in addition to reactivation of chick β-galactosidase immunoprecipitation and electrophoretic studies were carried out. The results of immunoprecipitation studies (Fig. 7) indicate that about

one third of the total β-gal activity was not precipitated by the antiserum and this thus represents chick β-gal activity. Of the two thirds of the β-gal activity which is human β-gal only a part can be ascribed to the expected residual activity of the I-cell fibroblasts (Fig.7 and Table 5).

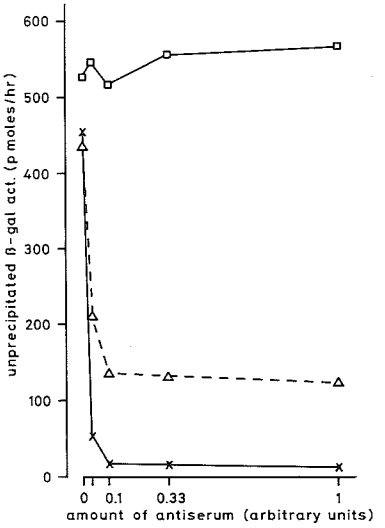

Fig.7 Immunoprecipitation of β-galactosidase in an homogenate of I-cell fibroblasts fused with chick erythrocytes, 8 days after fusion by an antiserum raised in rabbits against human placenta β-galactosidase. □——□ chick fibroblasts; **X——X** C5RO; △---△ I-cell x chick erythrocytes, 42% HET.K., 24% ERY.

The electrophoretic patterns of β-galactosidase are shown in Fig. 6B. The human fibroblasts show a two band pattern (lane 4) which is distinguishable from the broad apparently single band of chick fibroblasts and chick red blood cells (lane 5 and 3). The bands of activity of the I-cell-chick fusion are situated at the positions of both chick and human enzymes (lane 2). An increased activity at the human position is seen, when compared with the I-cell homokaryons (lane 1), in lane 1 and 2 almost equal amounts of proteins are used (79 µg and 84 µg respectively). We can conclude that human as well as chick β-gal activities are found in the I-cell-chick heterokaryons as a result of expression of N-acetyl glucosamine-1-phosphate transferase.

Expression of glucose-6-phosphate dehydrogenase

In control human fibroblasts the level of G6PD activity is around 12 µmoles/hr/mg protein. By comparison control chick fibroblasts have 55-65% of this level, and chick red blood cell activities are found from 0 - 2%. Two different G6PDD deficient human cell strains, with residual activities around 4% (EXP I) and 20% (EXP II) respectively, were fused with chick erythrocytes in order to see if there was re-activation of the chick gene coding for the constitutive cytosol enzyme G6PD. No increased G6PD activity was detected in the human-chick heterokaryons when compared with the human homokaryons (Table 6).

Table 6.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAYS ON CELL HOMOGENATES AFTER FUSION OF CHICK ERYTHROCYTES AND FIBROBLASTS OF PATIENTS WITH G6PD ACTIVITY

Exp.	Days After Fusion	$G6PD^-$ x ERY			$G6PD^-$ x $G6PD^-$
		% HET.K.[2]	% ERY[3]	G6PD act[1]	G6PD act[1]
I	2	76	49	0.5	0.6
	4	76	49	0.6	0.6
	8	75	45	0.7	0.7
	15	60	34	0.7	0.7
II	8	65	34	2.5	2.6
	15	68	34	2.2	2.4
	23	38	16	2.5	2.3
	30	35	17	1.8	1.9

Control Cultures	G6PD act[1]	
	EXP I	EXP II
Human Fibroblasts	11.9	13.0
Chick Fibroblasts	8.1	7.1
Chick Red Blood Cells	0.3	0.0

1) G6PD act in µmoles G6P/hr/mg protein

2) and 3) see Table 1.

DISCUSSION

The experiments reported here demonstrate that it is possible to switch on the synthesis of a number of lysosomal enzymes of chick origin and carry them through to the state of functional enzymes in heterokaryons with chick erythrocytes, even though we know that this processing involves a complex pathway of post-translational events. For instance the mRNA for such enzymes would be expected to include the characteristic hydrophobic leader sequence to expedite transfer of the molecule to the intracisternal space (27), where it is exposed to the subsequent proteolytic and glycosylation steps (for review see 13). The evidence from the fusion experiments with I-cell fibroblasts suggests that the glycosylation and mannose phosphorylation can be adequately generated. The retention of both chick and human β-galactosidase suggests that chick N-acetyl glucosamine-1-phosphate transferase is capable to restore the deficient processing of human lysosomal enzymes.

The generation of α-glucosidase is rapid and efficient since the level rises to 140% of the expected normal levels of chick at 15 days after fusion. The lower level of β-galactosidase activity (20% of control chick levels) is comparable to the order of regeneration of the cytosol enzyme HGPRT (28), while the expression of β-hexosaminidase does not reach significant proportions over the period of the time of these experiments. The same applies to the cytosolic enzyme G6PD. This observed variety in enzyme expression may find an explanation at all different levels of enzyme realization (29). A lower level of enzyme expression can be due to an inefficient transcription of the coding sequences and/or a restricted supply of protecting proteins leading to an increased turnover of the synthesized mRNA. Electron microscopical studies (12,30) suggested that the erythrocyte nuclei were not fully reactivated because the interchromatin and perichromatin granules were seldom observed and the nucleoli remained fibrillar, lacking the granular components. Therefore it is possible that the differences in lysosomal enzyme expression are caused by different reactivation patterns of the respective genes.

There may also be a limitation in the amount of mRNA transported to the cytoplasm. Bramwell (6) demonstrated that only a small amount of

chick ribosomal RNA appears in the cytoplasm of the heterokaryons, although after the introduction of the chick nucleus in the heterokaryons a new polarization of the cytoplasmic organelles and a development of a Golgi apparatus in the vicinity of the chick nucleus is seen (12). The involvement of the nucleolus in the cytoplasmic expression of specific proteins, originally proposed by Harris in 1969 (3) has received strong support from in situ hybridization experiments by John (31) in which cDNA prepared from mRNA of different sources hybridizes preferentially with nucleoli.

With particular reference to the lysosomal enzymes reasons for the varying rates of appearance of new chick enzymes following fusion may be found at the translation and post-translation level. At least the biological half lifes of these enzymes, which are resultant on relative rates of biosynthesis and degradation, seem to differ widely; the biological half life of human β-galactosidase has been estimated at 10 days (32). In hamster fibroblasts that of β-glucuronidase is reported to be as short as 4 to 5 days; while arylsulphatase-A has a predicted half-life greater than 30 days (33). It might be that the rate of biosynthesis of β-hex is low and therefore the enzyme does not reach detectable level within 23 days after fusion. In addition it should be noted that a further constraint is placed on production of active β-hexosaminidase. While α-glucosidase and β-galactosidase are initially generated as monomeric precursors, which are subject to proteolytic cleavage to maturation, β-hexosaminidase is, in human at least, an oligomeric structure and it is necessary that a coherent association of subunits takes place. That this failure to produce a significant level of β-hex activity is due to some steps in the biosynthetic process, rather than an excessive degradation, is borne out by the experiments in which precursor form of chick β-hex was incorporated into mutant human fibroblasts and was stable for at least 6 days.

In conclusion we could demonstrate the reactivation of markers residing in the cytoplasmic organelles e.g. α-glucosidase and β-galactosidase in the lysosomes and indirectly measured N-acetyl glucosamine-1-phosphate transferase in the ER/Golgi region. The expression of lysosomal enzymes is of particular interest since it may allow us to follow the intracellular route of "de novo" synthesized enzymes, and to study

the species specificity of the various post-translational modification steps.

ACKNOWLEDGEMENT

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paper IV

INTERACTION OF HUMAN AND CHICK DNA REPAIR FUNCTIONS IN UV-IRRADIATED XERODERMA PIGMENTOSUM–CHICK ERYTHROCYTE HETEROKARYONS

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SUMMARY

Fusion of chick erythrocytes with human primary fibroblasts results in the formation of heterokaryons in which the inactive chick nuclei become reactivated. The expression of chick DNA repair functions was investigated by the analysis of the DNA repair capacity after exposure to ultraviolet (UV) irradiation of such heterokaryons obtained after fusion of chick erythrocytes with normal human or xeroderma pigmentosum (XP) cells of complementation groups A, B, C and D. Unscheduled DNA synthesis (UDS) in normal human nuclei in these heterokaryons is suppressed during the first 2–4 days after fusion. The extent and duration of this suppression is positively correlated with the number of chick nuclei in the heterokaryons. Suppression is absent in heterokaryons obtained after fusion of chicken embryonic fibroblasts with XP cells (complementation group A and C).

Restoration of DNA repair synthesis is found after fusion in XP nuclei of all complementation groups studied. It occurs rapidly in XP group A nuclei, starting one day after fusion and reaching near normal human levels after 5–8 days. In nuclei of the B, C and D group increased levels of UDS are found 5 days after fusion. At 8 days after fusion the UDS level is about 50% of that found in normal human nuclei. The pattern of UDS observed in the chick nuclei parallels that of the human counterpart in the fusion. A fast complementation pattern is also observed in chick fibroblast-XP group A heterokaryons resulting within 24 h in a UDS level comparable with that in chick fibroblast-normal human heterokaryons. In heterokaryons obtained after fusion of chick fibroblasts with XP group C cells UDS remains at the level of chick cells. These data suggest that reactivation of chick erythrocyte nuclei results in expression of repair functions which are able to complement the defects in the XP complementation groups A, B, C and D.

Cultured cells of xeroderma pigmentosum (XP) patients are sensitive to ultraviolet light (UV). In most XP cell strains this sensitivity is accompanied by decreased levels of unscheduled DNA synthesis (UDS) following UV exposure, suggesting that the genetic defect in XP is located in the cell's capacity to repair UV-induced DNA damage (for review see [1, 2]). Fusion of cells from different XP patients results in heterokaryons which in some combinations show normal repair activity due to genetic complementation [3]. Based on this type of analysis so far seven different complementation groups could be identified in xeroderma pigmentosum [4, 5].

Fusion of XP cells with animal cells may reveal the presence or absence of the normal counterpart of the defective XP gene product in animal cells and provides a tool for comparison of DNA repair processes in different eukaryotic systems. In the present study we have chosen chicken cells for several reasons. By using chick erythrocytes as parental cells in the fusion the heterokaryons can be readily identified. Moreover, a contribution of the chick genome to the repair capacity of the heterokaryons could possibly be recognized by its dependency on the reactivation pattern of the chick nuclei. In 1972 Darżynkiewicz & Chelmicka-Szorc [6] did not find restoration of repair DNA synthesis in XP-chick erythrocyte heterokaryons 1 and 7 days after fusion. Paterson et al. [7] studied the repair of UV-induced pyrimidine dimers in XP (C group)-chick fibroblast heterokaryons and found that in the dark dimers were not removed from the DNA of the XP nuclei.

These studies suggest that chicken cells may not possess a gene product which is able to complement an XP defect (most probably the XP-C group defect). This finding is in accordance with other observations by Paterson et al. [8] suggesting that excision repair of thymine dimers is impaired in embryonic chick fibroblasts. Recently published data by Wade & Lohman [9] however, indicate that embryonic chick fibroblasts do perform dimer excision, albeit with kinetics that differs from that in human cells. By combining these three pieces of information it became interesting to compare the repair capacities of heterokaryons obtained after fusion of chick erythrocytes with XP cells of different complementation groups.

Our experiments showed that with all XP complementation groups tested the XP-chick erythrocyte heterokaryons performed repair DNA synthesis upon reactivation of the chick nuclei. The kinetics of repair depended on the complementation group of the XP cells.

Table 1. *Human cell strains used*

Strain	Nature	UV-induced UDS (% of control)
C5RO	Normal human	100
C7RO	Normal human	100
XP25RO	XP(A)[a]	0–5
XP11BE	XP(B)	5
XP4RO	XP(C)	10–15
XP21RO	XP(C)	10–15
XP3NE	XP(D)	10–25

[a] The letters in parentheses represent the complementation groups.

MATERIALS AND METHODS

Cell strains

The primary human fibroblast strains used in this study are shown in table 1. The level of unscheduled DNA synthesis is also mentioned. These strains were grown in Ham's F10 medium supplemented with 10–15% fetal calf serum (FCS) and antibiotics.

Blood was collected from the allantoic vessels of 12–13 days old chick embryos, centrifuged for 10 min at 4°C (300 g) in FCS. The pellet containing the erythrocytes was suspended in solution A (0.13 M NaCl, 2.7 mM KCl, 14 mM Na HCO_3 and 10 mM D-glucose).

Primary embryonic chick fibroblasts were a generous gift of Dr M. H. Wade (Medical Biological Laboratory, TNO, Rijswijk). Their origin has been described earlier [9]. The chick fibroblasts (passage 3–6) were grown in Ham's F10 medium supplemented with 5% FCS and antibiotics.

Cell fusion

Human fibroblasts and chick erythrocytes were mixed in a ratio 1:25. Cells of this mixed suspension were incubated with 250 HAU/ml β-propiolactone-inactivated Sendai virus. After 5 min at 4°C and 20 min at 37°C the cells were suspended in medium containing 10% FCS and seeded in Petri dishes provided with a coverslip and incubated at 37°C in humidified air with 5% CO_2. After 3 h the cultures were rinsed twice with solution A and incubated in F10 +6% FCS.

In fusions with chick embryonic fibroblasts the parental cells were labelled with latex beads of different size in order to identify the heterokaryons after fusion. The procedure has been published recently by Matsukuma et al. [10]. The fusion was performed with 50 HAU/ml Sendai virus.

UV-irradiation

UV-irradiation (predominantly 254 nm) of the washed and drained cultures was performed with a Philips TUV low pressure mercury tube (15 W) at an incident exposure rate of 0.7 J/m²/sec.

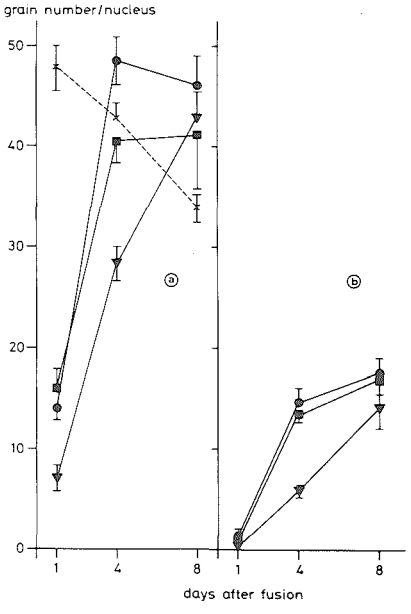

Fig. 1. Unscheduled DNA synthesis in heterokaryons obtained after fusion of chick erythrocytes with normal human fibroblasts (C5RO). The cells were exposed to a UV dose of 38 J/m² at the times indicated on the abscissa. UDS in (*a*) human nuclei; (*b*) chick nuclei. ×—×, Unfused human cells; ●—●, heterokaryons with two human and one chick nuclei; ■—■, one human and one chick nucleus; ▼—▼, one human and two chick nuclei.

Measurement of unscheduled DNA synthesis

The cultures were incubated in medium with [³H]-thymidine (10 μCi/ml; sp. act. 2 Ci/mmol) for 1 h before and for 2 h after UV irradiation and fixed in Bouin's fixative after rinsing in saline.

Autoradiographic preparations were made using either Kodak AR10 stripping film or Ilford K2 emulsion, exposure time was 5–15 days. After development and fixation the cells were stained with hematoxylin-eosin and prepared for counting. In each preparation the grains over the nuclei in at least 25 heterokaryons were counted. Nuclei in S phase, which had more than 100 grains, were excluded from counting.

RESULTS

UDS in normal human fibroblast–chick erythrocyte heterokaryons

The interaction between chick erythrocyte nuclei in the process of reactivation and normal human nuclei in terms of repair DNA synthesis was studied after fusion of normal human fibroblasts (C5RO) with chicken erythrocytes. The cells were exposed to a relatively high dose of UV irradiation (38 J/m²), 1, 4 and 8 days after fusion. UDS was measured in heterokaryons having different ratios of human to chick nuclei. The data for human and chick nuclei are presented separately in fig. 1*a* and *b*.

The UDS measured in the non-fused human cells in the population slightly decreases during the 8 days of growth. This decrease is often observed when cell populations become densely packed in a confluent stage of the culture. In comparison with the UDS level in the non-fused human cells UDS in the human nuclei in heterokaryons is extensively suppressed 1 day after fusion. At 4 days after fusion suppression is still apparent in heterokaryons containing one human and two chick nuclei, whereas the normal human level of repair is observed in the heterokaryons with the 1:1 and 2:1 human erythrocyte nuclei ratio. Chick nuclei in heterokaryons (fig. 1*b*) do not show UDS 1 day after fusion.

Repair is clearly observed 4 days after fusion and has reached about maximal levels at that time. In heterokaryons with one human and two erythrocyte nuclei the restoration of repair activity seems to be delayed.

These results indicate that the interaction between the human and chick nuclei is determined by a chick component which exercises suppression on human gene activity during the first days after fusion and a human component that allows, possibly in combination with reactivated chick functions, repair activity in chick nuclei at later times after fusion.

UDS in XP–chick erythrocyte heterokaryons

The results of two independent experiments in which XP25RO cells of complementation group A were fused with chick erythrocytes are presented in figs 2 and 3. The fusion with XP(C) cells is represented by fig. 4. The data given in figs 1, 2 and 4 were obtained in one experiment which allows comparison of UDS levels.

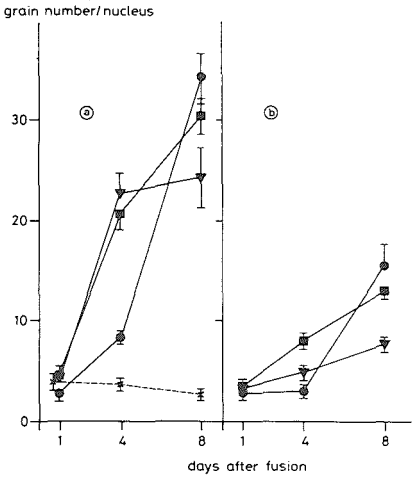

Fig. 2. Unscheduled DNA synthesis in heterokaryons obtained after fusion of chick erythrocytes with XP cells of complementation group A (XP25RO). For conditions and symbols, see fig. 1.

Figs 2 and 3 clearly show that UDS is restored in XP(A) nuclei after fusion with chick erythrocytes. In the experiment of fig. 3 some repair activity exceeding the residual level is already seen after 1 day, it increases in both experiments, reaching about normal human levels 8 days after fusion (compare figs 1 and 2).

The rate of repair seems to be dependent on the ratio of human to chick nuclei, being slowest in heterokaryons with two human and one chick nuclei. This is the reverse of the gene dosage relationship in fusions of normal human cells with chick erythrocytes (fig. 1*a*). UDS is also observed in the chick nuclei 4 and 8 days after fusion with XP group A cells (figs 2*b*, 3*b*), and follows a similar pattern as in the human nuclei in the heterokaryons. This suggests the action of a common repair system in both types of nuclei.

Restoration of DNA repair measured in UDS is also observed in heterokaryons obtained after fusion of XP(C) cells (XP4RO) with chick erythrocytes (fig. 4). One day after fusion UDS in the XP nuclei is below the residual level found in the non-fused cells, indicating a similar suppression effect as seen in normal human–chick eryth-

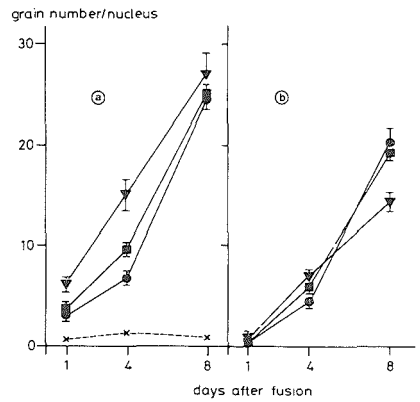

Fig. 3. Unscheduled DNA synthesis in heterokaryons obtained after fusion of chick erythrocytes with XP cells of complementation group A (XP25RO). This figure and fig. 2 represent two similar independent experiments. For conditions and symbols, see fig. 1.

rocyte heterokaryons (fig. 1*a*). After 4 days the level of UDS does not exceed the residual level and at 8 days a clear restoration of UDS is observed, which is about 50% of the level seen 8 days after fusion of normal human, or XP(A) cells with chick erythrocytes. Also the chicken nuclei perform UDS 8 days after fusion, the level being dependent on the human gene dosage.

These data suggest that, in contrast to the observation of Darżynkiewicz & Chelmicka-Szorc [6], interaction of XP and chick erythrocyte factors in the process of reactivation may result in restoration of UDS in XP as well as in chick nuclei. The rate of restoration after fusion with XP(A) cells is much faster than after fusion with XP(C) cells.

Besides a suppressive effect during the first days after fusion the chick nuclei clearly supply one or more factors that circumvent the repair defect in the XP nuclei. In the preceding experiments the cells were exposed to a relatively high dose of UV (38 J/m²). At that dose the excision repair system in normal human cells is saturated and the colony-forming capacity of unfused cells is negligible. The use of this high UV dose allows a comparison of the data with those of Darżynkiewicz et al. [6], who exposed the cells to 100 J/m² UV. In order to investigate DNA repair in the heterokaryons under conditions of cell survival in the next series of experiments the cells were exposed to only one-tenth of the dose (3.5 J/m²). In these experiments we compared cell strains from four different XP complementation groups with respect to the UDS induced by UV in binucleated XP-chick erythrocyte heterokaryons.

The results are presented in fig. 5. UDS was measured at 4, 5 and 8 days after cell fusion. In all fusions UDS levels are found in the human nuclei which clearly exceed the residual UDS observed in the non-fused parental cells in the same preparations (fig. 5*a*). As in the previous experiments the

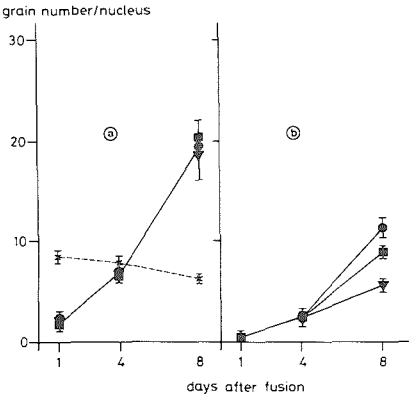

Fig. 4. Unscheduled DNA synthesis in heterokaryons obtained after fusion of chick erythrocytes with XP cells of complementation group C (XP4RO). For conditions and symbols, see fig. 1.

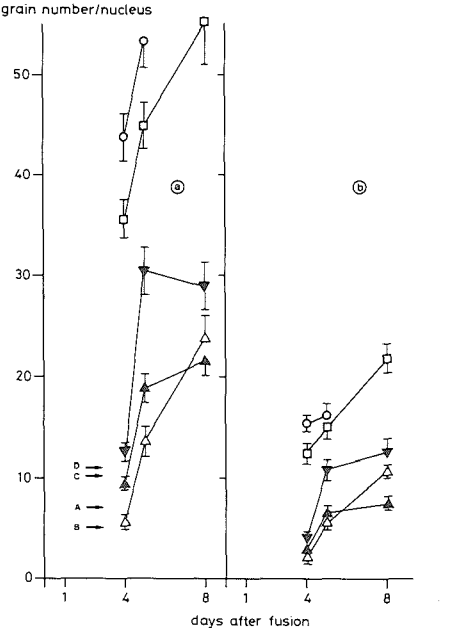

Fig. 5. Unscheduled DNA synthesis in binucleated heterokaryons obtained after fusion of chick erythrocytes with normal human and XP cells of different complementation groups. The cells were exposed to a UV dose of 3.5 J/m² at the times indicated on the abscissa. UDS in (*a*) human nuclei; (*b*) chick nuclei. Fusion with ○—○, normal human cells (C7RO); □—□, XP group cells (XP25RO); △—△, B group cells (XP1IBE); ▼—▼, C group cells (XP4RO); ▲—▲, D group cells (XP3NE). The arrows indicate the residual UDS levels in unfused XP cells of the different complementation groups.

XP(A) nuclei show a fast restoration of repair DNA synthesis reaching about normal human repair activity 4 days after fusion. At that time repair in nuclei of the B, C and D group is at the level of the non-fused XP cells. A sharp rise in UDS is seen in these nuclei after the 4th day reaching a plateau at the 5th day after fusion. Even after 8 days UDS in the B, C and D group nuclei is still about 50% of the UDS in normal human cells. These differences in repair level are also observed in the chick nuclei in the heterokaryons (fig. 5*b*).

UDS in human–chick embryonic fibroblast heterokaryons

In order to investigate the interaction of human and chick repair functions in a system in which suppression is not expected to occur, chick embryonic fibroblasts were fused with normal human and XP(A) and XP(C) cells. UDS measured in heterokaryons 24 h after fusion with normal human fibroblasts reached a level intermediate between that in human–human and chick–chick homokaryons (table 2). A similar level is found in heterokaryons 24 h after fusion of XP(A) cells with chick fibroblasts. This result suggests that the chick repair system provides a factor that is able to replace the human A factor completely. In the fusion with XP(C) cells a different pattern is obtained. At 24 and 48 h after fusion UDS in heterokaryons is performed at the level of the chick–chick homokaryons (table 2).

DISCUSSION

Fusion of normal human fibroblasts with chick erythrocytes resulted in suppression of repair DNA synthesis in the human nucleus when the heterokaryons were exposed to UV irradiation 24 h after fusion. The extent and duration of the suppressive effect increased with an increasing ratio of erythrocyte to human nuclei in the heterokaryons, which suggests that the suppression is exerted by chick-specific factor(s). This finding may be related to earlier observations of Carlsson et al. [11] and Darżynkiewicz et al. [12, 13] who described inhibition of RNA synthesis in the mammalian nucleus shortly after fusion with chick erythrocytes. By using protease inhibitors Darżynkiewicz et al. [12, 13] presented evidence that the suppression of RNA synthesis is due to the action of chicken proteins which in the process of reactivation of the chick nucleus become degraded by proteolytic enzymes in the heterokaryons. Although RNA synthesis is slightly suppressed in HeLa nuclei 24 h after fusion with chick erythrocytes [13], UDS reached normal levels when UV exposure was performed at that time after fusion [6]. This observation is in contrast with our findings with primary human fibroblasts. In order to clarify this apparent contradiction we have investigated the response of diploid fibroblasts and HeLa cells in more detail [15].

Table 2. *Unscheduled DNA synthesis in dikaryons exposed to 10 J/m² UV after fusion of human with chick fibroblasts*

Strains	Time after fusion (hours)	UDS (grains/nucleus) homokaryons		Heterokaryons Human/chick
		Human	Chick	
C5RO/chick fibroblasts	24	83.1 (3.0)[a]	34.1 (2.6)	56.7 (3.7)
XP25RO/chick/fibroblasts	24	4.6 (0.4)	40.7 (2.5)	50.6 (3.8)
XP21RO/chick fibroblasts	24	16.0 (0.6)	34.7 (1.5)	30.3 (1.7)
XP21RO/chick fibroblasts	48	15.4 (0.7)	35.5 (1.5)	34.1 (2.3)

[a] S.E.M.

Our experiments clearly demonstrated that restoration of DNA repair activity occurs in XP nuclei after fusion of XP cells with chick erythrocytes. It was found with all 4 XP complementation groups studied, group A, B, C and D, albeit with different kinetics. These results are in contrast with the data published earlier by Darżynkiewicz et al. [6]. In the latter study the complementation group of the XP cell strain was not known. As most excision-deficient XP strains have now been assigned to the groups A, C and D it is likely that their strain can be compared with one of the strains used in our experiments. In the case of an XP(A) strain a high level of UDS would have been expected to occur 7 days after fusion, the largest time interval after fusion studied by Darżynkiewicz and co-workers. If their strain belonged to group C or D it may be possible that due to the low rate of restoration seen with these cells these authors have not been able to detect UDS under their experimental conditions.

The observations described above strongly suggest that the repair activity demonstrated in the XP-chick heterokaryons is the result of an interaction of human and chick functions. The simplest way to explain the kinetics of UDS recovery in the XP(A)-chick heterokaryons is to assume that the chick components which influence the UDS level are of two different classes: (*i*) suppressing factors, the presence of which is transient in the course of the reactivation process [11–13]; (*ii*) chick-specific repair enzymes, among which is the one that complements the XP defect. These repair factors presumably increase during the process of reactivation. This hypothesis predicts that early after fusion the rate of UDS will be limited by the suppressing chick factors. This is in line with the initial suppression of the residual level of UDS of group C in the XP4RO/chick heterokaryons (see fig. 4). Also, in heterokaryons made up from one human and two chick nuclei (human to chick ratio of 0.5) similar levels of UDS are seen early after fusion when nuclei with a normal and an XP(A) genotype are compared (figs 1*a*, 2*a*). However, with a human to chick ratio of 2, the relative amount of the suppressive factors is lower, so that soon after fusion with XP(A) cells the amount of complementing repair factor provided by the chick probably becomes rate-limiting. This could explain the reverse gene dosage dependence observed in XP(A)-chick heterokaryons compared to cells obtained after fusion with normal cells (see figs 1–3). At later times after fusion the suppressive effect is no longer rate-limiting and the UDS has reached a value that is comparable to or only slightly lower than the normal human level. At this moment repair systems are in operation which are composed of human and chick functions. The comparable UDS levels in the human nuclei at 8 days after fusion of chick erythrocytes with either normal or XP(A) cells suggests that the chick repair system provides a factor that can replace the defective human A factor completely. This assumption is supported by the data obtained with chick embryonic fibroblast-XP(A) heterokaryons (table 2), which show an identical repair activity, as seen in heterokaryons after fusion of chick fibroblast with normal human fibroblasts.

The kinetics of restoration of repair in the case of fusion of chick erythrocytes with XP(B), (C) and (D) cells is clearly different from that of XP(A) cells. It occurs at a much slower rate and the UDS does not reach the level seen after fusion with normal human cells in a period of 8 days after fusion. However, the differences between these three XP types (fig. 5) and the dependency of the UDS after 8 days in the erythrocyte nuclei on the amount of XP(C) nuclei in the heterokaryons (fig. 4) suggest that also in these fusions UDS is the result of interaction of human and chick

repair functions. A low dose of 3.5 J/m^2 does not saturate the excision repair system in normal human cells. Therefore, it seems likely that in the heterokaryons with XP(B), (C) or (D) nuclei the chick component(s) required for restoration of repair are rate-limiting.

Experiments of Wade et al. [9] suggest that excision of dimers in chick fibroblasts occurs at a much lower rate than in human fibroblasts. This difference between human and chick fibroblasts is also observed in the fusion experiments presented in table 2, the UDS level in chick fibroblasts being about 40–50% of that in human fibroblast. In the fusion with XP(C) cells the chick-XP(C) heterokaryons and chick–chick homokaryons show similar levels of UDS. This finding also suggests that the chick factor that complements the defective human C factor is rate-limiting in these heterokaryons. The experiments with the XP(B), (C) and (D) cell strains (fig. 5) indicate that the chick repair factors which complement the B and D defects are rate-limiting as well.

It is interesting to note that in XP(A) chick erythrocyte heterokaryons complementation is already apparent at 1 day after fusion (fig. 3). This is at an early stage of reactivation of the nucleus when in most heterokaryons nucleolus formation is not yet detectable in the erythrocyte nucleus. So it appears that the chick factor that complements the XP(A) defect is readily produced when the reactivation process starts. It is tempting to speculate that this factor plays a role in the modification of chromatin structure in order to facilitate replication of DNA and/or transcription. A similar function can be assumed for this factor in a DNA repair pathway which requires modification of the chromatin structure in order to facilitate the accessibility of repair enzymes. Evidence for such a function of the A group factor has been postulated earlier by Mortelmans et al. [14] on different grounds.

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paper V

REPAIR DNA SYNTHESIS IN HETEROKARYONS DURING REACTIVATION OF CHICK ERYTHROCYTES FUSED WITH HUMAN DIPLOID FIBROBLASTS OR HELA CELLS

E. Van der Veer & D. Bootsma

SUMMARY

Suppression of unscheduled DNA synthesis (UDS) after exposure to ultraviolet light in the human nuclei results when diploid human fibroblasts are fused with chick erythrocytes. The suppression is positively correlated with the number of erythrocyte nuclei in the heterokaryons with a maximal effect at 36 hours after fusion. Evidence is presented that this suppression is due to lowered levels of the enzymes involved in UDS as a result of inhibition of the RNA synthesis by chick components.

No suppression of UDS is detected in the human nuclei of the HeLa-chick erythrocyte heterokaryons. In HeLa cells the amount of incorporated (^{3}H) uridine is about 10 times higher than the amount in the normal diploid fibroblasts, and the relatively small inhibitory influence of the chick components will therefore not lead to a limitation of the enzymes involved in UDS in the HeLa-chick erythrocyte heterokaryons.

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INTRODUCTION

In previous studies of repair DNA synthesis (unscheduled DNA synthesis, UDS) after ultraviolet (UV) irradiation in diploid human fibroblast-chick erythrocyte heterokaryons we found suppression of UDS in the human nucleus under the influence of chick nuclei at 24 hours after fusion. A stimulation of repair activity in the chick nuclei is detected at later times after fusion (1). Darzynkiewicz and Chelmicka-Szorc (2) investigated DNA repair after UV-irradiation in heterokaryons obtained after fusion of HeLa cells and chick erythrocytes. Their data suggest that in these heterokaryons UDS is not suppressed in the HeLa nuclei at 24 hours after fusion (2) although RNA synthesis was found to be inhibited (3). This is in agreement with the work of Carlsson et al. (4) who used rat epithelial cells. Reactivation of the chick erythrocyte nuclei may involve the release of suppressors ordinarily confined to the erythrocyte nucleus with subsequent redistribution of these suppressors among all the nuclei of the heterokaryons (3,5). These suppressors may be hydrolysed either in transit between the nuclei or within the nuclei by intracellular proteases after which RNA synthesis increases again.

In the present report we investigate this different pattern of UDS in human fibroblast and HeLa nuclei, partnered with chick erythrocyte nuclei in more detail and studied a possible relationship between suppression of UDS and inhibition of RNA synthesis.

MATERIALS AND METHODS

Cells

Human HeLa-S_3 cells and a primary human fibroblast strain (C5RO) are grown in Ham's F10 medium supplemented with 4% foetal and 4% newborn calf serum and antibiotics. The cells were free of mycoplasma as judged by the method of Chen (6). The chick erythrocytes were obtained by collecting blood from the allantoic vessels of 11-13 days old embryos, as described earlier (1).

Cell fusion

Heterokaryons were prepared by fusing chick embryonic erythrocytes with either HeLa cells or human fibroblasts, using ß-propiolactone inactivated Sendai virus (200 - 300 HAU/ml) as previously described (1). To obtain heterokaryons with different ratios of human and chick nuclei the human cells and the erythrocytes were mixed in a ratio of 1:25. Six hours after fusion the cultures were washed extensively to remove the non-fused erythrocytes.

Measurement of unscheduled DNA synthesis

UV-irradiation (predominantly 254 nm) of the washed and drained cultures was performed with a Philips TUV low pressure Mercury tube (15 Watt) at an incident exposure rate of 0.7 $J/m^2/sec$. The cultured were labelled with either (^3H) thymidine (10 µCi/ml; spec.act. 22 Ci/mmol) or deoxy (5-^3H) cytidine (10 µCi/ml; spec.act. 24 Ci/mmol; The Radiochemical Centre Ltd., Amersham). Before exposure to UV-light (10 J/m^2) the cells were prelabelled for one hour in order to facilitate the distinction between nuclei performing S-phase DNA replication and UDS. After UV-irradiation the cells were labelled for two hours, rinsed twice with saline and fixed in Bouins fixative. Autoradiographic preparations were made using Ilford K2 emulsion and exposure times of 4-10 days. After development and fixation the cells were stained with May Grünwald-Giemsa. Grain counts were performed over nuclei of 25 cells for each experimental point.

Measurement of RNA synthesis

The cultures after fusion were labelled during 15 min. with (^3H) uridine either 0.5 or 5 µCi/ml (spec.act. 40 Ci/mmol; the Radiochemical Centre Ltd. Amersham) rinsed twice with saline and fixed in Bouin's fixative. Autoradiographic preparations were made as described above. The number of grains over the nuclei of at least 25 cells were counted.

RESULTS

UDS in human fibroblast-chick erythrocyte heterokaryons

At various time intervals after fusion of C5RO human fibroblasts with chick erythrocytes the cultures were exposed to a dose of 10 J/m^2 UV. UDS was measured with (^{3}H) thymidine labelling for 2 hours starting immediately after UV exposure. The results of grain counts over human and chick nuclei in heterokaryons containing different ratios of human and chick nuclei are presented in Fig. 1. A decrease of UDS is observed in the human nuclei in the heterokaryons, with a

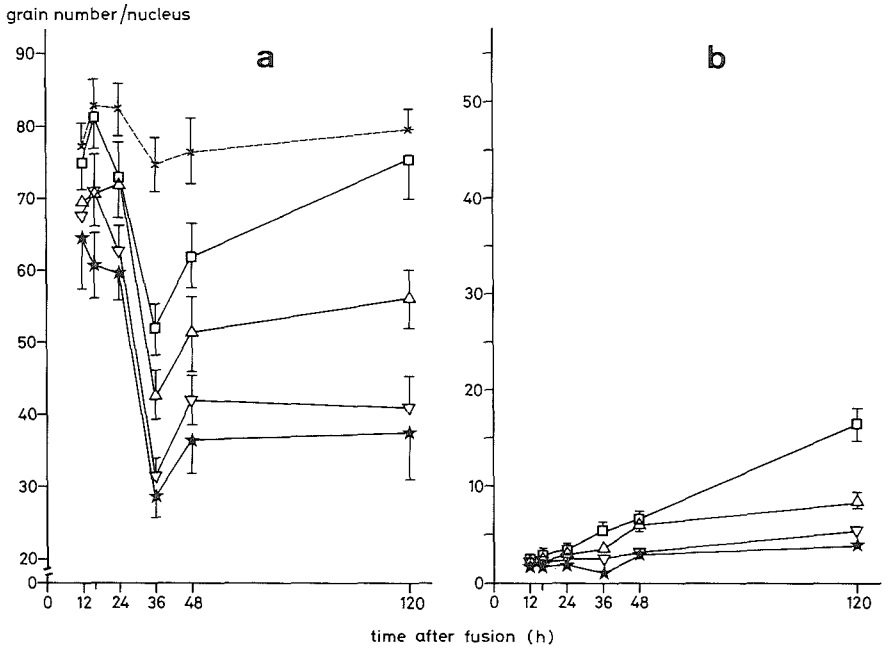

Fig. 1 Unscheduled DNA synthesis in heterokaryons obtained after fusion of diploid human fibroblasts (C5RO) with chick erythrocytes. The cells were exposed to a UV dose of 10 J/m^2 and 10 µCi/ml (^{3}H) thymidine is used for pré- and post-labeling. The times of fixation are indicated on the abscissa. a) UDS in the human nuclei, b) UDS in the chick nuclei. X---X , unfused human cells; □—□, heterokaryons with 1 human and 1 chick nucleus; △—△ , heterokaryons with 1 human and 2 chick nuclei; ▽—▽ , heterokaryons with 1 human and 3 chick nuclei; ✱—✱ , heterokaryons with 1 human and 4 chick nuclei.

maximal effect at about 36 hours after fusion when compared with the unfused human nuclei (Fig. 1a). The extent of suppression correlates with the number of chick nuclei in the heterokaryons. At five days after fusion suppression is still apparent in heterokaryons which have two or more chick nuclei; whereas the normal human level of repair is observed in heterokaryons with one human and one chick nucleus. The chick nuclei in the heterokaryons (Fig. 1b) do not show detectable UDS levels during the first 24 hours after fusion. At 36 hours UDS is detected in the heterokaryons with 1 human and 1 erythrocyte nucleus and increases steadily when irradiation is carried out at later times after fusion. The reactivation of UDS in heterokaryons with two or more chick nuclei seems to be delayed.

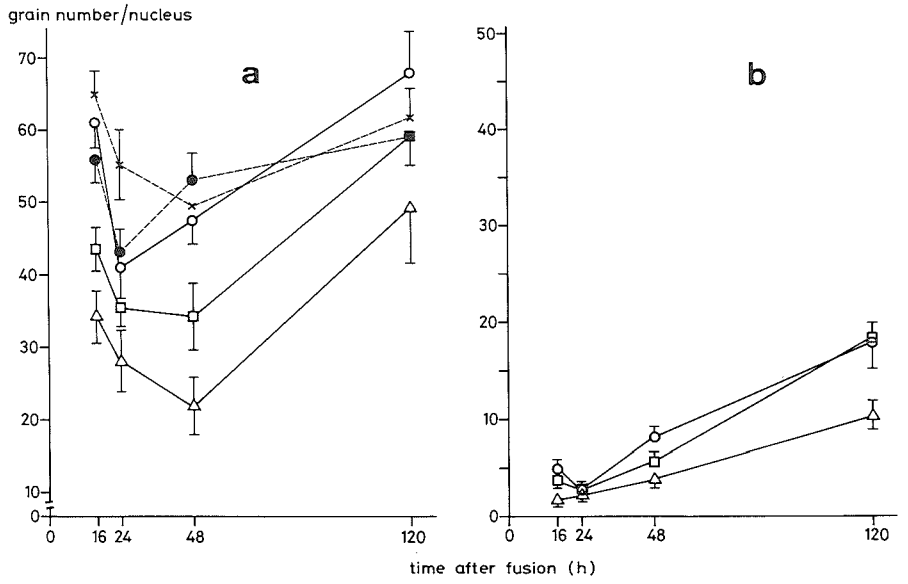

Fig. 2 Unscheduled DNA synthesis in heterokaryons obtained after fusion of diploid human fibroblasts (C5RO) with chick erythrocytes, labelled with 10 µCi/ml (^{3}H) deoxycytidine and exposed to a UV dose of 10 J/m^2. The times at fixation are indicated on the abscissa. a) UDS in the human nuclei, b) UDS in the chick nuclei. X---X , unfused human cells; ●--● , homokaryons with two human nuclei; O—O, heterokaryons with 2 human and 1 chick nuclei; □—□ , heterokaryons with 1 human and 1 chick nucleus; △—△ , heterokaryons with 1 human and 2 chick nuclei.

A similar experiment was carried out in which (^{3}H) deoxycytidine in stead of (^{3}H) thymidine was used to measure UDS (Fig. 2). The results indicate that the suppressive effect on UDS is independent of the DNA label used in these experiments. A slight decrease of the UDS level is also observed in the unfused mononuclear human cells and in the human homokaryons. This suppression which varies in extent and duration in different experiments is probably due to the fusion and seeding procedures at the beginning of the experiment.

UDS in HeLa-chick erythrocyte heterokaryons

The results of an UDS experiment in which HeLa cells were fused with chick erythrocytes exposed to 10 J/m^2 UV at different time intervals after fusion and labelled with (^{3}H) thymidine are presented in Fig. 3. The data given in Fig. 1 and Fig. 3 were obtained in the same

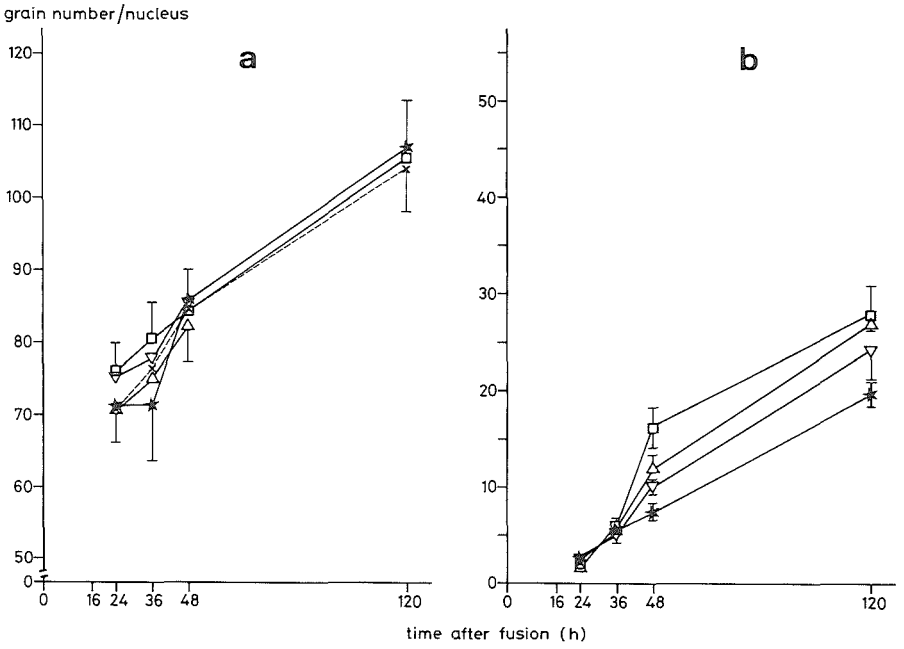

Fig. 3 Unscheduled DNA synthesis in heterokaryons obtained after fusion of HeLa cells with chick erythrocytes, labelled with 10 µCi/ml (^{3}H) thymidine. Conditions and symbols as in Fig.1

experiment, which allows comparison of the UDS levels. Unfused HeLa cells and heterokaryons with different ratios of HeLa and chick nuclei showed identical UDS patterns. A gradual increase in UDS level is observed in all HeLa nuclei regardless of whether they are present in mononuclear cells or in heterokaryons. This increase is probably due to the flattening of the HeLa nuclei after seeding which results in the enlargement of the nuclear surface. At 24 hours after seeding the mean surface area is 114 μm^2 and this is increased to 176 μm^2 at 120 hours. Such an increase has not been observed in the human fibroblasts.

RNA synthesis in heterokaryons after fusion of human cells with chick erythrocytes

In order to investigate a possible relationship between suppression of UDS and inhibition of RNA synthesis (^3H) uridine incorporation was determined at different time intervals after fusion of chick erythrocytes with either human fibroblasts or HeLa cells. To get a countable number of grains the C5RO-chick erythrocyte population was labelled with 5 µCi/ml and the HeLa-chick erythrocyte population with 0,5 µCi/ml. Within the first 24 hours after fusion of chick erythrocytes with C5RO cells, all human nuclei showed a low incorporation of (^3H) uridine (Fig. 4a). An increase is observed after 24 hours reaching a constant level after 36 hours in unfused fibroblasts. The human nuclei in the heterokaryons reach a lower level at 36 hours and this level is dependent on the number of erythrocyte nuclei in the heterokaryon. The low incorporation in unfused fibroblasts during the first 24 hours suggests an inhibitory effect on the RNA synthesis due to the treatment of the cells during fusion and seeding procedures. This inhibition may have overcast a suppressive effect exerted by the presence of the chick erythrocyte nuclei in the heterokaryons in that period. The latter suppression is clearly indicated at 36 hours after fusion and remains present until the end of the experiment. In other experiments suppression is no longer apparent in heterokaryons that have one human and one chick nucleus at 8 days after fusion (data not shown). The (^3H)-uridine incorporation pattern in the human nuclei in

the HeLa experiment is different from that in the fibroblast experiment (Fig. 4b). Although a gradual increase in (^{3}H) uridine labelling is observed during the first 48 hours in unfused HeLa cells and in homokaryons, a chick erythrocyte dependent suppression is already indicated at 16 hours after fusion. A fast recovery is evident after 36 hours, resulting in about normal incorporation levels at 72 hours. The gradual increase observed in unfused HeLa cells and in the homokaryons parallels the increase found in the UDS experiment with HeLa cells (Fig.3a).

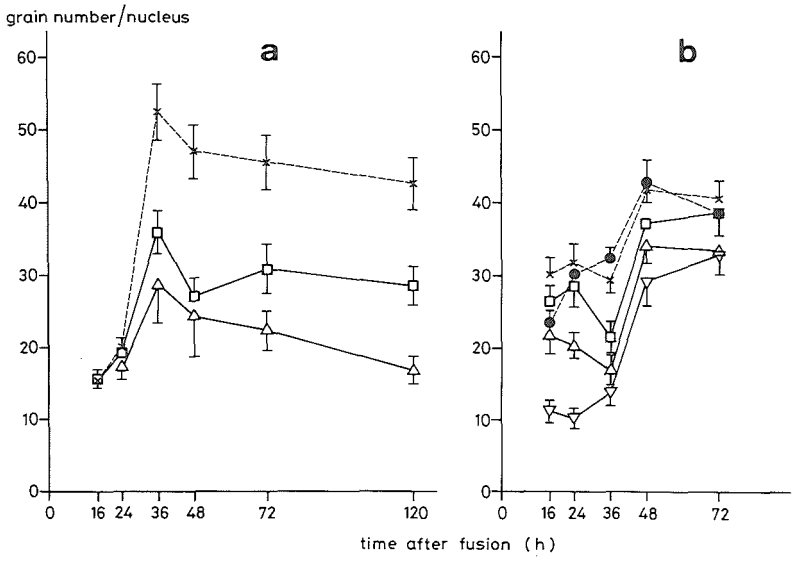

Fig. 4 (^{3}H) Uridine incorporation in the human nuclei of heterokaryons obtained after fusion of either C5RO or HeLa cells with chick erythrocytes for 15 minutes before fixation. The times of fixation are indicated on the abscissa. a) C5RO - chick erythrocyte fusion, labelled with 5 µCi/ml (^{3}H) uridine; b) HeLa-chick erythrocyte fusion, labelled with 0.5 µCi/ml (^{3}H) uridine. Symbols as in Fig. 1.

DISCUSSION

When diploid human fibroblasts fused with chick erythrocytes are exposed to UV-irradiation and either (^{3}H) thymidine or (^{3}H) deoxycytidine is used as precursor, suppression of UDS in the human nuclei results. The maximal effect is at about 36 hours after fusion. The extent and duration of the suppressive effect increases with an increasing ratio of erythrocytes to human nuclei in the heterokaryons, suggesting that suppression is exerted by chick-specific factors. These factors do not influence the UDS in HeLa nuclei after fusion with erythrocytes, over the 5 days following fusion. This is in accordance with Darzynkiewicz (2) who found the same level of UDS for all fused and unfused HeLa nuclei after 24 hours. During the first 24 hours after fusion of fibroblasts with chick erythrocytes about normal levels of DNA repair were observed in the human nuclei (Fig. 1a), whereas RNA synthesis is markedly inhibited during that period (Fig. 4a). These results suggest that the DNA repair capacity in these nuclei is independent of the RNA synthesis. Experiments carried out in our laboratory, in which cytoplasts derived from normal human fibroblasts were fused with UV-exposed xeroderma pigmentosum (XP) cells, have shown that these cytoplasts are able to correct the XP defect during a period of about 20 hours (7). These cybridization experiments indicate that the repair functions are maintained in the cytoplasts in the absence of RNA synthesis for about 20 hours. The initially normal UDS levels observed in the fibroblast-chick erythrocyte heterokaryons, therefore, indicate that the chick-specific suppressive factors do not interfere directly with DNA repair replication. It seems likely that at longer time intervals after fusion de novo synthesis of repair enzymes is required to maintain the repair capacity of the human nuclei in the heterokaryons. Due to the inhibitory effect of chick components on the RNA synthesis the amount of repair proteins may become rate limiting in the human fibroblast-chick erythrocyte heterokaryons. The absence of suppression of UDS in the heterokaryons containing HeLa nuclei can simply be explained by the 10 fold difference observed in the amount of incorporated (^{3}H) uridine when the HeLa cells are compared with the human fibroblasts.

The relatively small inhibitory effect on the RNA synthesis in the HeLa-erythrocyte heterokaryons most probably does not result in rate limiting levels of repair proteins in these heterokaryons.

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