

CELLULAR RETINOIC ACID BINDING PROTEIN:
CLONING AND EXPRESSION OF THE GENE

KLONERING EN EXPRESSIE VAN HET GEN CODEREND VOOR
CELLULAIR RETINYLZUUR-BINDEND EIWIT

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. C.J. RIJNVOS
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 20 MAART 1991 OM 15.45 UUR

DOOR

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Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam.

Het onderzoek werd mogelijk gemaakt door financiële steun van de Nederlandse Kankerbestrijding, Stichting Koningin Wilhelmina Fonds.

I keep six honest serving men
They taught me all I knew
Their names are What and Why and When
And How and Where and Who.

Rudyard Kipling, *Just-So Stories*

Voor J.J. en H.C.E.

In dank voor jullie nimmer aflatende steun



Contents

I. General introduction	7
1. The process of cellular differentiation	7
2. Embryonic pattern formation	9
3. Biological activities of retinoic acid	12
4. Molecular mechanisms of retinoic acid action	15
5. Scope of the thesis	18
II. cDNA cloning and characterization of mouse genes that are induced upon differentiation of an embryonal carcinoma cell line.	25
III. Preferential expression of cellular retinoic acid binding protein in a subpopulation of neural cells in the developing mouse embryo	51
IV. The cellular retinoic acid-binding protein is expressed in tissues associated with retinoic acid-induced malformations	67
V. Embryonal carcinoma-derived cell lines expressing different levels of cellular retinoic acid-binding protein exhibit differential responsiveness of the retinoic acid receptor β gene to induction by retinoic acid.	89
VI. Analysis of the promoter region of the cellular retinoic acid-binding protein gene: transcriptional regulation requires the presence of enhancer elements	99
VII. Isolation of human cellular retinoic acid-binding protein gene sequences and their localization relative to the acute promyelocytic leukemia-associated breakpoint on human chromosome 15	112
VIII. General discussion	125
1. Identification of genes that are transcriptionally induced following differentiation of a teratocarcinoma-derived cell line.	125
2. Implications for the nature of MES-1 cells in terms of normal embryonic development.	126
3. The biological function of CRABP.	127
4. The role of RA in embryonic development.	129
5. Molecular mechanisms of RA action.	131
6. Regulation of RA concentrations within the embryo; a role for CRABP?	132
7. Concluding remarks.	133
Summary	139
Samenvatting	141

Curriculum Vitae	143
Dankwoord	145
Abbreviations	149

Chapter I

GENERAL INTRODUCTION

A single cell, the fertilized egg, starts to cleave and multiply. The new cells rearrange themselves, and start to differentiate into a variety of cell types. The differentiated cells are organized into tissues and organs, thus creating body form and structure. Still the cells multiply. The embryo increases in size, and a new organism is generated.

The miraculous, yet commonplace event of embryonic development has held a particular fascination for scientists throughout all ages. The greek philosopher Aristotle was the first to crack open a chicken egg on each day of its 3-week development period, to observe the evolution of a thin band of cells into an entire bird, and to note the formation of the major organs. Starting with Aristotle, many embryologists have described the phenomena that can be observed during embryonic development.

Although embryology is obviously a very old science, real progress towards an understanding of how development actually occurs was not made until the last few decades. Indeed, embryonic development is a very complicated process in that it accomplishes the diverse functions of differentiation, morphogenesis and growth. The intricacies of the developmental processes and the difficulties involved in obtaining sufficient amounts of specific embryonic cells or tissues to allow scientific analysis have severely impeded scientific progress.

1. The process of cellular differentiation

Observing an organism, it becomes apparent that different cell types express different proteins. Being derived from one and the same fertilized egg, each of these cell types must contain the same genomic information. Development, then, involves the differential expression of specific genes at specific times and places (Morgan, 1934). The problem of developmental genetics becomes: How is the expression of genetic information regulated so that cells become different?

Research into the molecular mechanisms of cellular differentiation was facilitated by the discovery that certain cultured cell lines can be induced to differentiate *in vitro*. In this respect embryonal carcinoma (EC) cells, the undifferentiated stem cells of teratocarcinomas, are of special interest (for review, see Stevens and Pierce, 1975; Martin, 1980). A teratocarcinoma is a very special type of tumor in that it is composed not only of neoplastic stem cells, but also of a variety of differentiated cells and tissues

at various stages of maturation. It is assumed that a teratocarcinoma arises by neoplastic transformation of an undifferentiated cell, which is pluripotent and may differentiate into derivatives of all three embryonic germ layers. During tumor development, a random array of differentiated structures is formed as some stem cells differentiate into cartilage, and others into nerve, muscle, glandular tissue, or other cell types. In general, these differentiated derivatives have a normal, non-malignant appearance. However, some stem cells may continue to proliferate in the undifferentiated state, thus forming nests of pluripotent EC cells interspersed in the disorganized mixture of differentiated derivatives. The continued proliferation of this undifferentiated stem-cell population is responsible for the malignant properties of teratocarcinomas. In cases where the stem cells cease to proliferate because they differentiate or die, the tumors become benign.

Teratocarcinomas may arise spontaneously in the gonads, but can also be induced experimentally. When early mouse embryos are grafted to extra-uterine sites, they will develop into teratocarcinomas (Stevens, 1970). Several EC cell lines originating from such experimentally induced tumors have been established *in vitro*, and can be maintained in an undifferentiated state in culture (Martin, 1975; Graham, 1977). Under suitable culture conditions (Martin and Evans, 1975; Nicolas *et al.*, 1976; McBurney, 1976) or after return to ectopic sites *in vivo* (Kleinsmith and Pierce, 1964), EC cells can differentiate into virtually all cell types observed in the original tumor. When cultured EC cells are injected into the blastocoelic cavity of normal embryos, they may even join normal development to form a chimeric embryo (Brinster, 1974; Mintz and Illmensee, 1975; Papaioannou *et al.*, 1975; Illmensee and Mintz, 1976; Dewey *et al.*, 1977). Contribution of EC cells to a wide variety of normal tissues has been reported (Mintz and Illmensee, 1975; Mintz and Cronmiller, 1978), demonstrating that cultured EC cells retain some of the developmental potential of early embryonic stem cells.

EC cells thus resemble cells of the early embryo in their pluripotency and they also share biochemical, morphological and antigenic properties with pluripotent embryonic cells (Martin, 1980). The possibility to grow large numbers of EC cells and study their differentiation *in vitro* has made the murine teratocarcinoma a popular model system for investigating normal embryonic development. In tissue culture, EC cells show little tendency to differentiate during exponential growth. Changes in local cell density (aggregation) and/or the addition of chemical stimuli may result in the formation of various differentiated derivatives (Artzt *et al.*, 1973; Martin and Evans, 1975). Especially the vitamin-A derivative retinoic acid (RA) is a potent inducer of EC cell differentiation (Strickland and Mahdavi, 1978). By using the appropriate cell line and differentiation inducing protocol, a particular line of differentiation can be studied.

P19 is an example of a pluripotent EC cell line that can give rise to derivatives corresponding to all three germ layers, namely ectoderm, endoderm and mesoderm

depending on the culture conditions (McBurney *et al.*, 1982). When the cells are grown as a monolayer, RA induces the formation of endodermal derivatives. Upon aggregation in the presence of RA, differentiation into neurectoderm can be observed. Following aggregation with dimethylsulfoxide (DMSO), P19 EC cells are induced to form mesodermal derivatives, including rhythmically contracting muscle cells. Clonal cell lines have been isolated representing these three lines of P19 EC cell differentiation (Mummery *et al.*, 1985; 1986). Being derived from one parental cell line, these cell lines will be much alike in genotype. However, they are very different with respect to morphology and expression of various biochemical markers, and therefore provide a model system for the study of the different lines of differentiation.

When EC cells are induced to differentiate, they undergo dramatic morphological alterations, reflected in qualitative and quantitative alterations in the expression of large numbers of genes (reviewed by Astigiano *et al.*, 1989). Many structural extracellular matrix and cytoskeletal proteins, such as fibronectin, laminin, lamin, entactin, collagen, and keratin have been reported to be differentially expressed. Also secreted proteins may be induced in some differentiation pathways, as has been demonstrated for plasminogen activator, α -fetoprotein, and apolipoprotein E. Putative regulatory genes showing changes in expression include the homeobox-containing genes and several proto-oncogenes, such as *c-fos* and *c-myc*. Most of the shifts in gene expression occurring during EC cell differentiation are assumed to be associated with the differentiated phenotype, but some must be involved in the regulation of the differentiation process *per se*. If the alterations in gene expression taking place during EC cell differentiation indeed resemble those occurring during early embryonic development, identification of such differentially expressed gene sequences may provide insight in the molecular processes taking place during embryogenesis.

2. Embryonic pattern formation

Although the study of cellular differentiation may provide information on what gene sequences are induced or repressed as the cells undergo phenotypical changes, and may even reveal what regulatory genes are involved in these processes, it cannot explain how the ordered pattern of differentiated tissues that constitute a developing embryo is generated. An early embryonic cell, explanted to an extra-uterine site, out of its normal context, will develop into a teratocarcinoma. An embryonal carcinoma cell, injected into a mouse blastocyst, will participate in the formation of a normal embryo. These findings implicate that the major difference between a teratocarcinoma and a normal embryo lies

not in the intrinsic properties of the composing cells, but in the presence or absence of regulatory signals that govern pattern formation.

According to Lewis Wolpert's theory of positional information (Wolpert, 1969), the cells within a developing system have their position specified with respect to one or more reference points or "organizing regions". This positional information is then fixed in a more or less stable form as the cell's positional value. Each cell interprets its positional value according to its genome and developmental history by differentiating in a particular direction. The idea was that positional value is established by having a graded concentration profile of a morphogen, a signal substance emitted from the organizing region across the embryonic field. A morphogen is thus a particular kind of inducing factor characterized by the evocation of different cellular behaviours at different concentrations. The positional information theory revitalized interest in the search for biochemical signal molecules, and a number of potential morphogens has been identified in a variety of developmental systems, including the Coelenterates (Schaller *et al.*, 1989), the slime mould *Dictyostelium* (Williams, 1988), the amphibian blastula (Smith, 1989), and the *Drosophila* embryo (St Johnston *et al.*, 1989).

Valuable information was also obtained from the study of limb development in the avian embryo. Vertebrate limbs derive from a common ancestral form and exhibit a remarkable degree of homology. As illustrated in Figure 1, the limb is a complex organ with an asymmetric pattern of parts. Basically, the bones of the forelimb consist of a humerus adjacent to the body wall, a radius and ulna in the middle region, followed by bones of the wrist and digits. The orientation can be described in terms of three orthogonal axes: (1) the anteroposterior axis, as in the line between the thumb and little finger, (2) the dorsoventral axis, as in the line between the upper and lower surfaces of the hand, and (3) the proximodistal axis, as in a line connecting the shoulder and the fingertip.

Most insights into limb development have come from studies of the experimentally accessible chicken embryo. The limb arises from a small bud that protrudes from the embryonic flank (Newman, 1988). It comprises a thin peripheral layer of ectoderm enclosing a loosely packed mass of mesoderm. As a consequence of mesenchymal-ectodermal interactions, the mesenchyme induces the epithelium at the limb bud margin to thicken, which results in a columnar pseudostratified epithelium known as the apical ectodermal ridge (AER) (Saunders, 1948). The AER is essential for limb outgrowth, since removal will result in a limb that is truncated (Saunders, 1948). After the AER has been formed, the limb bud rapidly elongates distally, and the mesenchyme differentiates in a coordinate fashion into an intricate pattern of cartilage, bone, muscles and other tissues that constitute the fully developed limb.

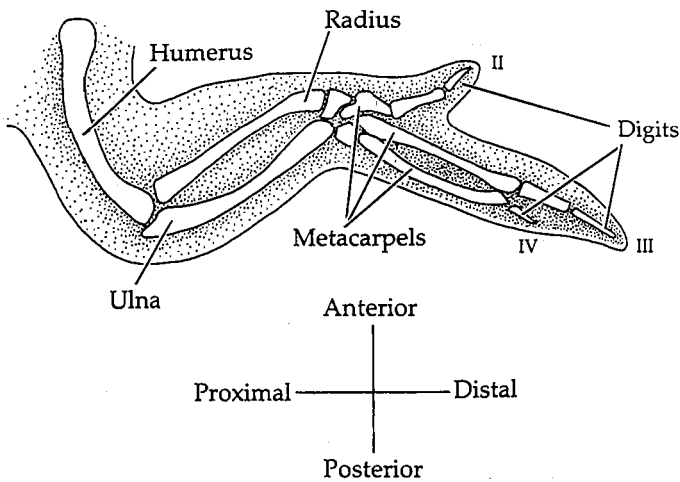


Figure 1
Skeletal pattern of the chick wing. Digits are numbered according to convention: 2, 3, 4.

Part of the posterior limb bud mesenchyme seems to function as an organizing region (Saunders and Gasseling, 1968). Transplantation of this so-called zone of polarizing activity (ZPA) to an anterior position in a host limb bud may result in the formation of additional digits from host tissue in mirror-image symmetry to the original limb - that is, a 43234 pattern may develop from the manipulated limb bud instead of the normal digit pattern 234 (see Figure 2). Tickle *et al.* (1975) performed a series of such grafting experiments and analyzed the results in terms of a model in which the ZPA emitted a morphogen that diffuses into the limb bud and thereby forms a concentration gradient spanning the anteroposterior limb axis. Cells next to the ZPA sense a high morphogen concentration which ultimately translates into the formation of a digit 4. Cells further away from the ZPA, i.e. more anterior, will be exposed to decreasing concentrations of morphogen and become digits 3 and 2, respectively. Implantation of a second ZPA opposite the endogenous one generates a mirror-symmetrical U-shaped gradient and this will lead to a mirror-symmetrically duplicated limb pattern.

In an attempt to identify the nature of the putative morphogen, Tickle *et al.* (1982) developed a method whereby either chemicals or extracts of polarizing region cells can be locally applied to the chick wing bud by being bound to implanted carrier beads. Because of its known effects on cell to cell communications and cell differentiation, retinoic acid (RA) was one of the chemicals tested for morphogenetic activity, and it was found that local application of this agent to the anterior margin of the limb bud mimics the effect of grafting a ZPA (Tickle *et al.*, 1982; Summerbell, 1983).

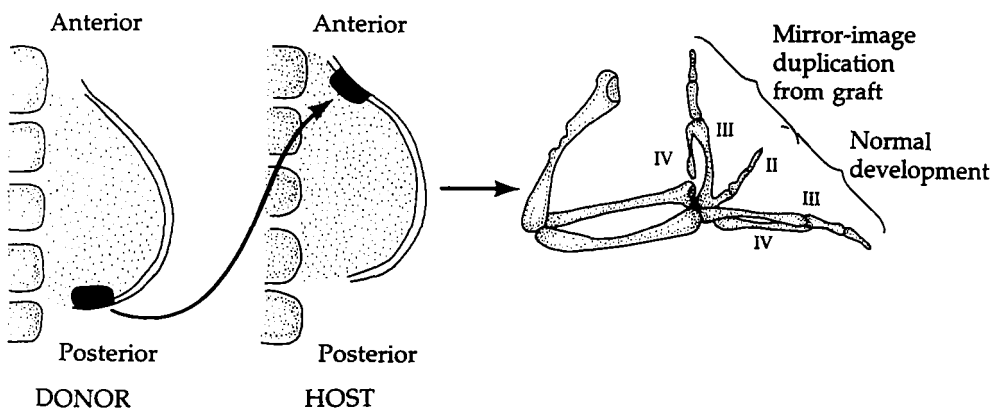


Figure 2

Formation of mirror-image duplications when a block of mesoderm from the posterior margin of the chick wing bud is transplanted to the anterior margin of a host wing bud. The resulting wing shows mirror-image duplications of digits 4 and 3; digit 2 is shared.

The possibility that RA may be related to the as yet unidentified inducer substance thought to be released by the ZPA inspired Thaller and Eichele to measure endogenous RA concentrations in developing chick limb buds (Thaller and Eichele, 1987). The authors demonstrated that, during embryonic stages capable of responding to ZPA grafts or RA administration, the chick limb bud contains endogenous RA in quantities known to be sufficient for induction of cellular differentiation *in vitro*. Even more exciting, the authors could show via bisection of the limb buds that RA forms a concentration gradient across the limb bud with a high-point in the posterior part, corresponding to the location of the ZPA. Thus, RA can induce extra structures and is present in the appropriate amounts and spatial distribution to be responsible for inducing the normal pattern of limb structures. This evidence strongly suggests that RA plays an important role in normal embryonic limb development, and possibly also in other morphogenetic events.

3. Biological activities of retinoic acid

Retinoic acid, a small lipid-soluble molecule of 300 Da, is derived from all-*trans*-retinol (vitamin-A alcohol or vitamin A₁). The biological importance of vitamin A and its derivatives, collectively referred to as the retinoids, in physiologic growth, visual function,

cell differentiation, and reproduction has long been recognized (for review, see Dicken, 1984). The adverse effects of hypervitaminosis A have been well known since their description in arctic explorers who had eaten raw polar bear livers. Adults who have acutely ingested large amounts of vitamin A frequently experience fatigue, malaise, anorexia, and headaches. More chronic symptoms include dry skin, fragile nails, sore lips, nosebleeds, weight loss, and bone and joint pain. Male patients may also complain of impotence (Silverman et al., 1987). However, not only excessive vitamin A ingestion, but also vitamin A deficiencies have deleterious effects (Wolbach and Howe, 1925). Animals fed a retinol-deficient diet fail to thrive, and eventually die. Less severe cases of vitamin A deficiency may result in night blindness, impaired spermatogenesis, and various epithelial abnormalities.

The physiological function of retinoids is not quite understood, but it is likely that most effects of hyper- or hypovitaminosis A can be explained by alterations in cellular differentiation. An exception to this is the function of vitamin A in the visual process, which has been well defined (Wald, 1968). Hippocrates already mentioned that consumption of liver may cure night blindness. Retinol is the precursor for all-*trans*-retinal, which contributes to the formation of the light-sensitive visual pigment rhodopsin.

One of the pathologic consequences of vitamin A deficiency is a dry and coarse skin. The cutaneous lesions, characterized by hyperkeratosis, are gradually normalized by a supply of retinol. Histopathological studies on retinol-deficient animals revealed in many tissues the replacement of the columnar and transitional epithelium by squamous, frequently keratinizing epithelial cells that multiply rapidly (Wolbach and Howe, 1925). These dramatic changes can be fully reversed by restoring a normal diet. Thus, vitamin A appears to be necessary for normal differentiation and maintenance of the differentiated state of epithelial tissues.

Retinoids have been extensively utilized in the therapy of neoplastic and hyperproliferative dermatoses. They are known to inhibit keratinization and alter cell maturation (Dicken, 1984). In the early 1960s Tretinoin (all-*trans*-retinoic acid) was tested for its therapeutic effect on basal cell carcinomas, actinic keratoses, and seborrhoeic warts (Stüttgen, 1986). When given orally, this drug demonstrated a significant degree of toxicity (Schumacher and Stüttgen, 1971). Therefore, a search for retinoids with fewer deleterious side effects was begun. 13-*cis*-retinoic acid, a naturally occurring vitamin A analogue marketed as isotretinoin, was shown to be very effective in the treatment of acne vulgaris (Bollag and Geiger, 1984). The synthetic retinoid Etretinate is primarily used for the treatment of psoriasis and disorders of keratinization (Orfanos, 1980). Ornithine decarboxylase has been shown to be intimately associated with the hyperproliferative lesions of psoriasis. Etretinate inhibits this enzyme and is

believed to exert its antipsoriatic effect by blocking the physiologic function of this protein (Lowe *et al.*, 1982).

RA is also applied as a therapeuticum in certain leukemic disorders. It has been shown that RA stimulates differentiation of the human promyelocytic leukemia cell line HL-60 and fresh acute promyelocytic leukemia (APL) cells (Breitman *et al.*, 1980, 1981). RA induces complete remissions in certain patients with APL without the development of marrow hypoplasia (Huang *et al.*, 1988), indicating that the antileukemic activity of RA *in vivo* is probably due to differentiation induction of the neoplastic cells.

The administration of retinoids for therapeutic purposes has revealed that vitamin A and its derivatives are potent teratogens (Kamm, 1982). In addition to their embryopathic effects (Lammer *et al.*, 1985) these medications lead to spontaneous abortion in about one third of exposed pregnancies (Shalita, 1988). Lammer and co-workers analyzed 154 pregnancies in which there had been fetal exposure to isotretinoin. The incidence of birth defects is very high: forty-six percent of the pregnancies taken to delivery resulted in the birth of a child with some abnormality. Most frequent were malformations of the central nervous system, congenital heart defects, occasionally combined with thymic defects, and craniofacial malformations, such as microtia/anotia, micrognathia, cleft lip and cleft palate. In experimental animals, fetal exposure to large amounts of retinoids may result in abnormal development of almost any organ system (Kochar, 1967, Geelen, 1979). The exact nature of the defect is dependent on the time of administration and the dose (Jelinek and Kistler, 1981; Goulding and Pratt, 1986; Birnbaum *et al.*, 1989; Abbott *et al.*, 1990). However, the same tissues as in the human isotretinoin syndrome are predominantly affected, indicating that the teratogenic effects of retinoids are exerted through a mechanism general to various vertebrate species.

The tissues that are generally affected by fetal exposure to excess vitamin A or RA share a common embryonic origin in that they are either derived from the neurectoderm or have received contributions from the cranial neural crest. This has led to the hypothesis that excess RA has an adverse effect on cephalic neural crest cells, possibly by disturbing normal neural crest cell migration (Thorogood *et al.*, 1982; Pratt *et al.*, 1987). This theory is supported by the observation that RA interferes with the cell-substratum adhesion of neural crest cells grown *in vitro* (Smith-Thomas *et al.*, 1987).

Taken together, the observed effects of RA on cellular differentiation and embryonic pattern formation suggest that RA exerts its biologic activities by mediating the expression of specific sets of genes (Chytil and Sherman, 1987). The fact that embryos actually contain appreciable amounts of RA points to RA as an endogenous signal molecule which plays an important role in normal development.

4. Molecular mechanisms of Retinoic Acid action

Most vitamin A is ingested as retinyl esters, the storage form of retinol. Within the intestinal lumen, these esters are hydrolysed to form retinol. Similarly, β -carotene and other dietary carotenoids may be biochemically modified to form retinol intraluminally (Silverman *et al.*, 1987). Once inside the blood stream, vitamin A is transported bound to retinol-binding protein (RBP), a polypeptide produced by the liver. The synthesis of this protein is influenced by plasma retinol-RBP concentrations (Dicken, 1984). After hepatic uptake, excess vitamin A is stored as the retinyl ester (Raica *et al.*, 1972; Dicken, 1984; Silverman *et al.*, 1987).

The amount of retinoic acid in the diet is negligible in comparison with retinol and its precursor carotenes. The liver appears to be able to form retinoic acid from retinol, but retinoic acid is not stored in this organ (Dowling and Wald, 1960). Retinoic acid may be found in a variety of tissues, including blood (De Leenheer *et al.*, 1982). Whereas retinol is transported in complex with a specific retinol binding protein, retinoic acid circulates within the vasculature bound to albumin (Leyden, 1988).

Within the cytoplasm, vitamin A-like compounds are complexed to highly specific binding proteins (reviewed by Chytil and Ong, 1987). Two of these, cellular retinal binding protein (CRALBP) and the interphotoreceptor or interstitial retinol binding protein (IRBP), are present only in visual tissue and presumably play a role in the visual function of vitamin A (Chader, 1982; Bridges, 1984). The other intracellular retinoid binding proteins, cellular retinoic acid binding protein (CRABP) and the two types of cellular retinol binding protein (CRBP), can be detected in a variety of normal and malignant cells and tissues (Ong *et al.*, 1982). These proteins belong to a family of small cytoplasmic lipid binding proteins that also includes liver and intestinal fatty acid binding proteins (FABPs) and myelin protein P2, and is commonly referred to as the myelin P2 family of proteins (Eriksson *et al.*, 1981; Sundelin *et al.*, 1985). Members of this protein family show a marked structural similarity, especially in their amino terminal regions (Sundelin *et al.*, 1985), and also exhibit a significant degree of homology at the DNA level (Wei *et al.*, 1987). In spite of their structural similarities, CRBP and CRABP exhibit striking binding specificities for retinol and RA respectively (Ong & Chytil, 1975; Jetten & Jetten, 1979).

Evidence for an involvement of CRABP in the effects of RA on differentiation has been provided by cell culture experiments. As mentioned earlier, a variety of cell lines can be induced to differentiate by treatment with RA. Striking correlations between the presence of the binding protein in the cell and its responsiveness to RA have been reported (Schindler *et al.*, 1981; Sherman *et al.*, 1981). Teratocarcinoma cells in which CRABP was detected differentiated when RA was added to the culture medium. Mutant

cells selected for their failure to differentiate in response to RA did not contain detectable CRABP. In addition, the binding affinity of various synthetic RA analogues to CRABP correlates remarkably well with their biological potency (Jetten and Jetten, 1979; Lotan, 1980; Sani *et al.*, 1984; Keeble and Maden, 1986). However, the above is contrasted by the human myelocytic leukemia cell line HL60, which is prompted to differentiate to mature granulocytes in the presence of RA, but appears to be deficient in CRABP (Breitman *et al.*, 1981; Douer and Koeffler, 1982).

It has been proposed that CRABP has a function in the transport of RA through the cytoplasm to the nucleus, where it is thought to exert its biologic effects (Jetten and Jetten, 1979; Takase *et al.*, 1986). This hypothesis was based on the observation by Jetten and Jetten that binding of RA to EC cell nuclear extracts is detected only in extracts prepared from cells that were preincubated with RA. This result seems to indicate that transfer of RA to its nuclear receptor sites can occur only after cytoplasmic interaction of RA with the cytoplasmic binding protein.

A major step forward in the understanding of the molecular mechanisms of RA action was the identification of a family of nuclear receptors for RA. Up till now, three different RA receptors (RARs) have been described in mouse and human, designated RAR α (Petkovich *et al.*, 1987; Giguère *et al.*, 1987), RAR β (de Thé *et al.*, 1987; Brand *et al.*, 1988), and RAR γ (Krust *et al.*, 1989). Multiple isoforms of these RARs may occur (Giguère *et al.*, 1990; Kastner *et al.*, 1990). An additional RAR, designated RAR δ , which is specifically expressed in regenerative tissues of the newt has been described by Ragsdale *et al.* (1989).

The RARs show a marked structural similarity to the superfamily of steroid/thyroid hormone receptors. Proteins belonging to this family contain functional domains for ligand binding, DNA binding, and trans-activation (see Figure 3). In the presence of the cognate ligand, these nuclear receptors are able to activate or inhibit transcription of specific genes by interacting with responsive elements located in the promoter region of these genes (reviewed by Evans, 1988). By analogy to steroid receptors, binding of RA to the RARs is thought to trigger the activation or repression of specific networks of genes. More direct evidence that the RARs may function as sequence-specific transcriptional activators was provided by the demonstration that RAR α can activate transcription at high levels through interaction with a previously isolated hormone responsive element for the thyroid hormone receptor (Umesomo *et al.*, 1988). Moreover, the rat growth hormone gene, which is known to be regulated by thyroid hormone, was found to be sensitive to RA in a pituitary cell line (Bedo *et al.*, 1989). These results suggest that RA and thyroid hormone could control overlapping gene networks and thus exert a concerted control on developmental events. Another apparent target of RA-mediated gene regulation is the RAR β gene itself (De Thé *et al.*,

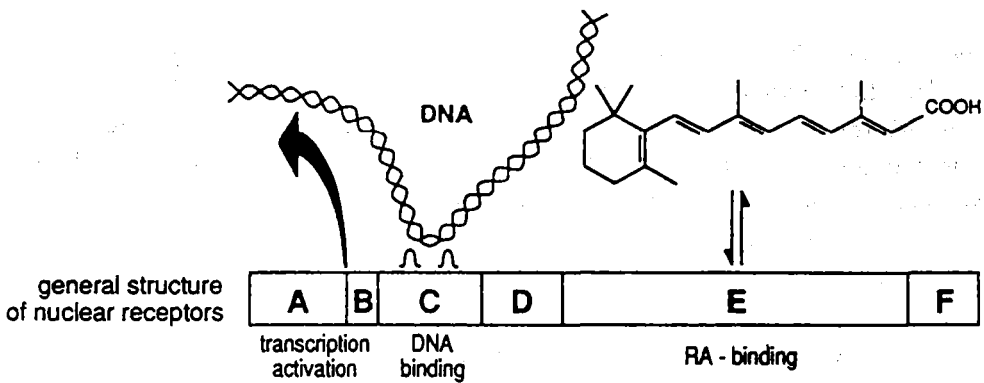


Figure 3

Schematic representation of the primary structure of a retinoic acid receptor, divided into six regions (A, B, C, D, E, and F) by analogy with the general structure of nuclear receptors.

1989; Song and Siu, 1989; Hu and Gudas, 1990). RNA analysis of hepatoma and embryonal carcinoma cells revealed that expression of RAR β increases in response to RA, indicating that RAR β is autoregulated. Indeed, a specific RA-responsive element has recently been identified in the promoter region of the RAR β gene (De Thé *et al.*, 1990).

The different RARs share extensive homology with one another, but are distinguished by their affinities for RA (Brand *et al.*, 1988). Moreover, *in situ* hybridization studies have shown that all three murine RARs exhibit unique spatiotemporal expression patterns during embryonic development (Dollé *et al.*, 1989; Ruberte *et al.*, 1990). Differential expression of the RARs, coupled to selective activation by different RA concentrations, could provide an explanation of how RA can elicit such a wide variety of biological effects.

5. Scope of the thesis

The work described in this thesis aims at the elucidation of mechanisms that govern cellular differentiation. To gain insight in these processes, molecular changes associated with differentiation of embryonal carcinoma cells were investigated. As similar differentiation events are assumed to occur during normal embryogenesis, this may contribute to the understanding of early embryonic development.

Chapter II describes the isolation and preliminary characterization of a number of cDNA clones corresponding to gene sequences that are induced upon differentiation of a mouse embryonal carcinoma cell line. After determination of the nucleotide sequences, we were able to establish the identity of four of the selected cDNA clones. One of these was found to encode the cellular retinoic acid binding protein CRABP. In view of the known involvement of RA in cellular differentiation and embryonic pattern formation, we decided to focus our attention on analysis of the CRABP gene. The *chapters III and IV* deal with the expression of CRABP during embryonic development. Our data show that the CRABP gene exhibits a spatio-temporally specified expression pattern in both mouse and chick embryos. A striking correlation is observed between CRABP expression and susceptibility to RA-induced malformations. In *chapter V*, tissue culture experiments will be described that provide a better understanding of the role of CRABP in RA-mediated signal transduction. Finally, we have started to study the regulation of CRABP gene expression. These experiments, which include a detailed analysis of the CRABP promoter region, will be presented in *chapter VI*.

The results presented in this thesis show that a gene that is induced upon differentiation of an EC cell line is also differentially expressed during embryogenesis. Our *in vivo* expression data point to an important role for CRABP in the regulation of RA mediated morphogenetic events.

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Chapter II

cDNA CLONING AND CHARACTERIZATION OF MOUSE GENES THAT ARE INDUCED UPON DIFFERENTIATION OF AN EMBRYONAL CARCINOMA CELL LINE

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Part of this chapter has been published as:

Vaessen, M.-J., Kootwijk, E., Bootsma, D., Westerveld, A., and Geurts van Kessel, A. *Cytogenet. Cell Genet.* 51, 45, 1989.

Abstract

The pluripotent mouse embryonal carcinoma (EC) cell line P19 can be induced to differentiate *in vitro* in response to certain culture conditions. The cell line MES-1 is a clonal derivative of P19 exhibiting mesoderm-like characteristics. Assuming that cellular differentiation is accompanied by changes in gene expression, we have screened a MES-1 cDNA library for gene sequences that are abundantly expressed in the differentiated cells, but not in the undifferentiated stem cells. Following this approach, we selected nine independent MES-1 cDNA clones. Expression of the corresponding genes was studied in various EC cell lines and their derivatives, and also in mouse embryos and adult tissues. On the basis of the observed expression patterns, five clones were selected for further analysis. Following determination of the nucleotide sequences, computer-assisted database comparison resulted in the identification of the genes corresponding to four of the selected MES-1 cDNA clones. The nature of these differentially expressed

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genes is discussed. In addition, the chromosomal localisation of two genes in mouse and/or human could be established using panels of suitable somatic cell hybrids.

Introduction

Mouse embryonal carcinoma (EC) cells, the undifferentiated stem cells of teratocarcinoma, are remarkably similar to the pluripotent cells of the early embryo. EC cells are often used as a model system for early mammalian development, allowing detailed study of differentiation *in vitro* (Martin, 1980). Stem cells from many cultured teratocarcinomas are capable of differentiating in response to certain culture conditions. The P19 EC cell line, for example, can give rise to derivatives of all three germ layers (McBurney et al., 1982). Treatment of P19 EC cells grown in monolayer with retinoic acid (RA) results in the generation of endodermal derivatives. Upon aggregation in the presence of RA, P19 EC cells form neurectodermal derivatives. Aggregation in the presence of dimethylsulfoxide (DMSO), leads to the formation of mesodermal derivatives, including spontaneously contracting muscle cells.

Differentiation of cells is generally accompanied by a large shift in the pattern of gene expression (Tocci et al., 1983). Identification of differentially expressed gene sequences has already provided valuable information about the molecular mechanisms involved in gene regulation (Brickell et al., 1983; Mason et al., 1986; Angel et al., 1987; Distel et al. 1987; Milbrandt, 1987). In mouse EC cells various differentiation pathways are marked by changes in the expression of particular genes (Paulin et al., 1981; Linder et al., 1981; Levine et al., 1984; Ikuma et al., 1986). Newly synthesized differentiation-specific proteins include structural proteins (e.g. laminin and collagen IV) (Strickland and Mahdavi, 1978), enzymes (e.g. plasminogen activator) (Strickland and Mahdavi, 1978; Strickland et al., 1980), and cell surface proteins (e.g. B2 microglobulin, H-2 antigen, growth factor receptors) (Rees et al., 1979; Croce et al., 1981; Heath et al., 1981; Mummery et al., 1986).

In this study, we describe the selection of cDNA clones corresponding to mRNAs present in the clonal cell line MES-1, a mesodermal derivative of P19 EC which was obtained after treatment of P19 EC cell aggregates with DMSO (Mummery et al., 1986). MES-1 exhibits a high degree of differentiation, and possesses several general properties common to mesodermal cells. As such, it may be expected to express gene sequences specific for this particular direction of differentiation.

Differential screening of a cDNA library, prepared from MES-1 poly(A)⁺ RNA has resulted in the isolation of 9 different cDNA clones, corresponding to gene sequences which exhibit a high level of expression in MES-1 as compared to P19 EC cells. We have

studied the expression of these genes in two unrelated EC cell lines and their various differentiated derivatives, as well as in adult and embryonic mouse tissues. Based on these expression studies, some of the MES-1 cDNA clones were selected for further analysis. The nucleotide sequences were determined and compared to known sequences via computer-assisted database search. This approach led to the identification of four genes that are induced upon differentiation of P19 EC to mesoderm-like derivatives. In addition, we have established the chromosomal localisation of two of these genes in mouse and/or human.

Materials and Methods

Cell lines and culture techniques

P19 and C1003 EC cells were cultured as described previously for PC13 (Mummery et al., 1984). Mesodermal differentiation of P19 was induced as described by McBurney et al. (1982) by aggregating cells for 5 days in the presence of 1% DMSO, then replating in tissue culture grade plastic dishes. RNA was isolated after 7 days. Cultures then consist of a heterogeneous mixture of fibroblast-like cells with areas of rhythmically contracting muscle; no EC cells were evident. The MES-1 cell line was isolated by single cell picking from an area of contracting muscle from DMSO-treated aggregates (Mummery et al., 1986). The Fib-9, IF8C8, and 5CB2E11 cell lines were cloned from a culture of C17-S1 (clone C1003) EC cells, cultured in serum-free medium for 2 days, followed by culture in serum-containing medium for a further 5 days. This induced formation of various mesodermal cell types, as described by Darmon and Serrero (1983).

The panels of mouse-hamster and human-hamster somatic cell hybrids used for the chromosomal localisation have been described (Hilkens *et al.*, 1986; Geurts van Kessel *et al.*, 1983). These cells were cultured according to standard procedures. The chromosomal constitution was deduced from cytogenetic and isoenzyme or DNA marker analyses. DNA extraction and checks for chromosomal content were carried out using the same batch of cells.

RNA isolation and blot hybridization

Total RNA was isolated from cell lines using the LiCl Urea method described by Auffray and Rougeon (1980). The same procedure was applied for the isolation of RNA from BALB/c mouse embryos and adult tissues.

For RNA blot analysis, total RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde, and transferred to nitrocellulose filters (Maniatis, 1982). DNA probes, labelled with ^{32}P using random priming as described by Feinberg and Vogelstein (1983) were hybridized to the RNA blots at 42°C in a buffer containing 9% dextran sulphate and 50% formamide. The filters were washed at 65°C, twice in 3xSSC, 0.1% SDS, and twice in 1xSSC, 0.1% SDS.

DNA isolation and blot hybridization

High molecular weight DNAs were extracted from cell lines according to standard procedures (Jeffreys and Flavell, 1977). Following cleavage with restriction enzymes, DNA fragments were separated by electrophoresis on agarose gels, and transferred onto nitrocellulose filters by Southern blot techniques (Southern, 1975). Hybridization of the DNA blots was carried out at 65°C in the presence of 9% dextran sulphate. Preparation of radioactive probes and washing conditions were the same as described for Northern blot analysis.

Construction of the cDNA library

Poly(A)⁺ RNA was purified from total cellular MES-1 RNA after two cycles of selection on an oligo (dT)-cellulose column (Aviv and Leder, 1972). cDNA synthesis was performed as described by Gubler and Hoffman (1983), using an oligo (dT) primer for reverse transcription of the RNA, followed by self-primed second strand synthesis. Double stranded cDNA was tailed with poly (dC), and annealed with PstI-digested poly(dG) tailed pBR322 (BRL). Transformation of *E.coli* RR1 resulted in a library of approximately 5×10^4 clones.

Differential colony hybridization.

About 10^4 cDNA clones were cultured separately in 96 well plates, which were used as master plates, and replica-plated onto a pair of genescreen-plus filters (NEN). Colony hybridization (Grunstein and Hogness, 1975) was carried out using single stranded (^{32}P) cDNAs from poly(A)⁺ RNA of P19 EC and MES-1 cells.

Nucleotide sequence analysis

For determination of the nucleotide sequence, fragments of the MES-1 cDNA inserts were subcloned into the plasmid vector pTZ18R or pTZ19R (Pharmacia). After infection

of the resulting recombinant clones with m13 helper bacteriophages, single stranded DNA was isolated. The DNA sequences were determined via the dideoxy chain termination method (Sanger *et al.*, 1977), using Sequenase (United States Biochemical, Cleveland, Ohio) according to instructions by the manufacturer.

Computer-assisted analysis of the DNA sequence was carried out on a Vax 8530 using the Staden operating system. The sequence databases of the European Molecular Biology Laboratory and the National Biomedical Research Foundation were searched for sequence homologies.

Isolation of a human probe homologous to the MES-1 cDNA clone BG12

The human genomic library CML-0, kindly provided by Dr. G. Grosveld, consists of circa 15 kb. DNA fragments, generated by partial digestion with MboII, that were inserted into the BamHI site of bacteriophage vector EMBL-3. Screening of this library using the BG12 cDNA insert as a probe in hybridization resulted in the isolation of several positive clones. Fragments of these human genomic DNAs were subcloned into plasmid vector pTZ18R. The subclone P900 contains a 900 bp. PstI fragment of human genomic DNA that hybridizes to part of the BG12 cDNA insert, and does not contain human repeat DNA, as evidenced by its lack of hybridization to ³²P-labelled total human genomic DNA fragments.

Results

Preparation and screening of a cDNA library from MES-1 cell RNA

In order to isolate cDNA clones corresponding to genes that are abundantly expressed in MES-1, but not in P19 EC cells, a MES-1 cDNA library was prepared. MES-1 poly(A)⁺ RNA was isolated and converted to double stranded cDNA, which was cloned into the PstI site of pBR322 by dC - dG tailing. The recombinant plasmids were used to transform *E.coli* RR1, yielding a cDNA library of circa 50.000 colonies. The estimated size of the cDNA inserts ran up to 600 bp. Approximately 10.000 colonies were picked and cultured separately in 96 wells plates. From these plates, identical replica filters were prepared. Each set of filters was hybridized to ³²P cDNA, prepared from either MES-1 or P19 EC poly(A)⁺ RNA. Initial screening yielded approximately 500 clones that hybridized to the MES-1 probe but not to the P19 EC probe. These clones were rescreened following the same procedure. In the rescreen, 144 clones were found to

hybridize reproducibly to the MES-1 probe only. These were submitted to further analysis.

To examine how many different RNAs were represented in the collection of 144 differentially hybridizing MES-1 cDNA clones, we examined the cloned inserts for sequence homology by cross hybridization. Plasmid DNA was isolated from small scale cultures of the 144 positive cDNA clones. Following digestion with PstI to separate the cDNA inserts from the plasmid vector DNA, the DNAs were submitted to gel electrophoresis. Multiple Southern blots were prepared from each gel, and hybridized to ³²P labelled plasmid DNA from separate positive clones, until the sequence homology of every clone in the collection was established. From this analysis we found that the selected MES-1 cDNA clones represented at least 13 different mRNAs.

RNAs corresponding to the selected cDNA clones

For characterization of the transcripts corresponding to the different MES-1 cDNA clones, total RNA from MES-1 and P19 EC cells was analyzed by Northern blotting procedures, using ³²P labelled plasmid DNA from the appropriate cDNA clone as a probe in hybridization (Figure 1). The Northern blot results showed that, in spite of the fact that no hybridization to ³²P labelled P19 EC cDNA was observed in the colony filter hybridization experiments, 4 of the selected clones could detect some minor expression in the P19 EC cells (e.g. Figure 1, panel D). These four clones were excluded from further analysis. The other clones (listed in Table 1) showed no detectable hybridization with P19 EC total RNA.

To investigate whether induction of the genes encoded by the selected cDNA clones is limited to the clonal cell line MES-1, or is characteristic for DMSO induced mesodermal differentiation of P19 EC, Northern blot analysis was carried out using RNA isolated from P19 EC cells aggregated in the presence of DMSO and subsequently replated. Cells were harvested when significant areas of contracting muscle cells were observed. As shown in Table 1, transcription was detected by all clones except clone BG12.

Transcription of the differentially expressed genes in different EC cell systems

In order to determine whether the selected MES-1 cDNA clones were able to detect transcripts in P19 EC derivatives representing the other embryonic germ layers, we performed Northern blot analysis using RNA isolated from EPI-7 and END-2. These two P19 EC-derived clonal cell lines exhibit neurectodermal and endodermal characteristics, respectively (Mummery et al., 1985).

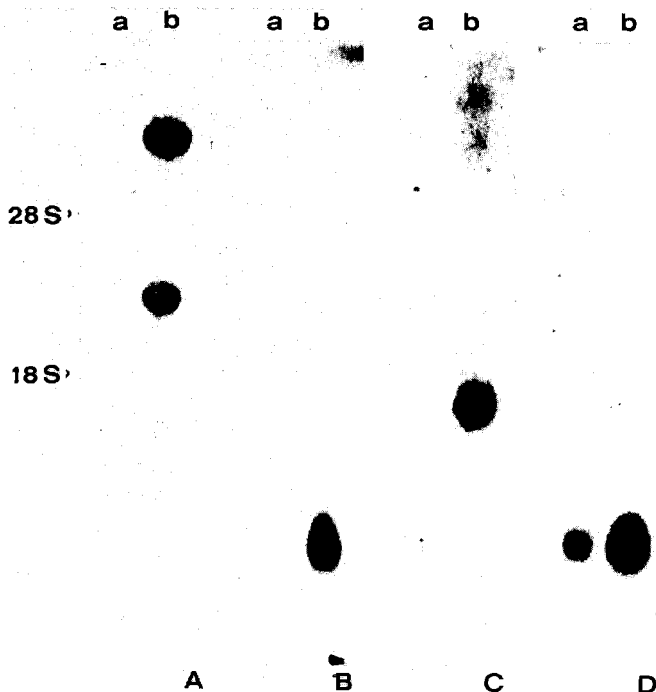


Figure 1

RNAs corresponding to the selected MES-1 cDNA clones. 20 μ g of total cellular RNA, isolated from P19 EC (lane a) or MES-1 (lane b) was analysed by Northern blotting, using the MES-1 cDNA clones AA2 (panel A), FE11 (panel B), EH12 (panel C), and EB12 (panel D) as probes in hybridization.

We also examined whether differential expression of the cloned genes is limited to the P19 EC cell system, or could be observed in another EC cell system as well. For this experiment, we chose C1003, a mouse EC cell line which differentiates to mesodermal cell types in response to temporary deprivation followed by re-addition of serum (Darmon and Serrero 1983). Thus, in contrast to MES-1 cells, the C1003-derived mesodermal cell lines Fib 9, 1F8C8, and 5CB2E11 (Mummery, C.L. and A. Piersma, unpublished) were not obtained as a result of chemical induction.

Table 1 summarizes the results of these Northern blotting experiments. mRNAs corresponding to clones AA2, BG12, and CA11 were not detected in EPI-7 and END-2, and appear to be specific for the mesodermal line of differentiation. The other clones correspond to genes expressed in all three P19 EC derivatives. Results obtained with the C1003 cell line and its mesodermal derivatives largely parallel those of the P19 EC cell

EC cell lines	MES-1 cDNA clones								
	AA2	BG12	EC2	CA11	EH12	FE11	CB5	EH2	EB5
P19 EC	-	-	-	-	-	-	-	-	-
P19+DMSO	+	-	+	+	+	+	+	+	+
MES-1	+	+	+	+	+	+	+	+	+
EPI-7	-	-	+	-	+	+	+	+	+
END-2	-	-	+	-	+	+	+	+	+
C1003	-	-	-	-	-	-	-	-	-
Fib9	+	+	+	-	+	+	+	+	+
IF8C8	+	±	±	-	+	+	±	-	±
5CB2E11	-	+	+	-	+	+	+	+	+

-: transcription not detected

+: readily detectable level of transcription

±: transcription detectable, but low as compared to +

Table 1

Expression of the selected MES-1 cDNA clones in various EC cell lines and derivatives

system. Only clone CA11 showed no detectable hybridization with RNA isolated from any of the C1003 derived mesodermal cell lines.

Transcription of the differentially expressed genes in mouse embryos and adult tissues

After establishing that the selected cDNA clones encode genes that are differentially expressed in various EC cell systems, we studied the presence of the corresponding mRNAs *in vivo*. Total cellular RNA was isolated from a variety of mouse organs. In view of a possible role of the cloned genes in embryogenesis, RNA was also extracted from mouse embryos at day 13, 16 and 19 *post coitum*, and from whole newborn mice. Northern blot analyses using the selected cDNA clones as probes in hybridization yielded the results summarized in Table 2. Clone EH2 detects transcription in liver, kidney, heart and spleen, whereas transcripts corresponding to EB5 are present in kidney, heart and

Tissues and embryos	MES-1 cDNA clones								
	AA2	BG12	EC2	CA11	EH12	FE11	CB5	EH2	EB5
Kidney	+	-	-	-	±	-	+	+	±
Heart	±	-	-	-	+	-	+	+	±
Testis	±	-	-	-	+	-	+	-	-
Thymus	±	-	-	-	+	-	+	-	+
Brain	+	-	-	-	±	-	±	-	-
Liver	+	-	-	-	±	-	±	+	-
Spleen	±	-	-	-	+	-	±	±	-
Muscle	-	-	-	-	+	-	+	n.d.	n.d.
Embryos:									
Day 13	-	-	-	+	+	+	+	+	+
Day 16	-	-	-	+	+	+	+	+	+
Day 19	-	-	-	±	+	+	+	+	+
Newborn	-	-	-	-	+	n.d.	+	n.d.	n.d.

-, +, ± : see Table I
n.d. : not determined

Table 2
Expression of the selected MES-1 cDNA clones in mouse embryos and adult tissues

thymus. Clones AA2, EH12 and CB5 hybridized more or less strongly with RNA from all tissues examined. Clones BG12, EC2, CA11, and FE11 showed no detectable level of transcription in the adult mouse organs tested in these experiments. Hybridization with embryonic RNA was observed with all clones except AA2, BG12 and EC2. Interestingly, the transcript corresponding to clone CA11 appears to diminish with the increasing age of the embryo, becoming undetectable in the newborn mouse.

Genomic hybridization and evolutionary conservation of selected MES-1 cDNA clones

We performed Southern blotting experiments to study the structures of the mouse genomic DNAs corresponding to cDNA clones AA2, BG12, CA11, and EC2. To establish whether the gene concerned is conserved during evolution, we also examined whether these mouse cDNAs showed cross hybridization with genomic DNAs from different

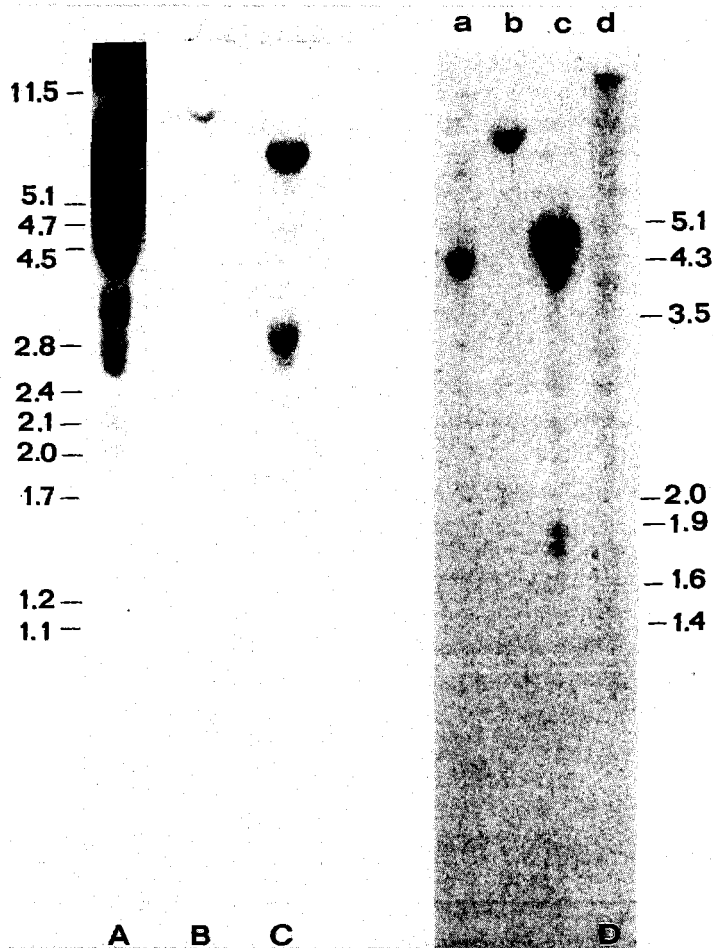


Figure 2

*Southern blot analysis of genes corresponding to four cloned cDNAs. Genomic DNA was isolated from mouse (panels A, B, C and lane a of panel D), Chinese hamster, (lane b), rat (lane c) and human (lane d), and digested with *EcoRI*. Southern blots were hybridized with *MES-1* cDNA clones AA2 (panel A), CA11 (panel B), EC2 (panel C), and BG12 (panel D).*

species.

Total genomic DNA from mouse, rat, hamster and man was digested with *EcoRI* and submitted to agarose gel electrophoresis. Nitrocellulose filter blots were prepared, and hybridized with each of the ^{32}P labelled cDNA inserts. Figure 2 shows some results of these Southern blot experiments. The genomic sequences hybridizing to clone AA2

were apparently repetitive, and were dispersed in the mouse genome. The other cDNA clones hybridized to single or a few copy genes. Clones BG12 and EC2 show a strong homology with rat, hamster, and human genomic sequences, even under stringent conditions of hybridization. Homology of clone CA11 with human genomic DNA is detectable only if relaxed hybridization conditions are applied. Clone AA2 showed no cross hybridization with human DNA.

Identification of selected MES-1 cDNA clones

Determination of the nucleotide sequence of the MES-1 cDNA clones was complicated by the fact that our MES-1 cDNA library consists of oligo(dT) primed cDNAs which were cloned into plasmid vector pBR322 by way of dC-dG tailing. This cloning strategy implies that the resulting cDNA inserts contain a dA/dT tail on the 3' side, and are flanked by dC/dG tails. The presence of these tails hampers accurate sequencing of the ensuing nucleotides according to the dideoxy chain termination method. To circumvent this problem, we have looked for restriction sites within the cDNA inserts, and used these to generate suitable subclones.

To separate the cDNA inserts from the plasmid vector DNA, all MES-1 cDNA clones had been subjected to digestion with *Pst*I. This had already revealed that the clones EC2 and EH12 contained internal *Pst*I sites. Following cleavage with an additional number of restriction enzymes, the cDNA inserts of clones BG12, CA11, and FE11 were found to contain *Eco*RI, *Rsa*I and *Pvu*II sites, respectively. These internal restriction sites were used to generate subclones in plasmid vectors pTZ18R or pTZ19R.

Making use of these subclones, the nucleotide sequence of five of the selected MES-1 cDNA clones was determined, whereupon computer-assisted database comparisons were carried out. Thus, the identity of four MES-1 cDNA clones could be established. As illustrated by Figure 3

, the newly determined nucleotide sequence of clone BG12 bears a strong homology to the mRNA sequence of a placental Calcium binding protein (pCaBP) (Jackson-Grusby *et al.*, 1987). The nucleotide sequence of clone EH12 corresponds to the mRNA sequence of mouse vimentin as published by Capetanaki *et al.* (1990). Figure 4 shows the optimal alignment, with the EH12 sequence on the upper line and the mouse vimentin mRNA sequence underneath. The third MES-1 cDNA clone that could be identified is clone FE11, which exhibits a strong homology to RL-14, an endogenous, soluble, dimeric β galactoside binding lectin (Clerch *et al.*, 1988). The FE11/RL-14 sequence alignment is illustrated by Figure 5. The newly determined nucleotide sequences of clone EC2, which is shown in Figure 6, was also compared with known sequences. However, this

```

          10      20      30      40      50      60
pCaBP  ACGGTTACCATGGCAAGACCCTTGGAGGAGGCCCTGGATGTAATTGTGCCACCTCCAC

          70      80      90      100     110     120
pCaBP  AAATACTCAGGCAAAGAGGGTGACAAGTTCAAGCTGAACAAGACAGAGCTCAAGGAGCTA
BG12   : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
          GCAAAGAGGGTGACAAGTTCAAGCTGAACAAGACAGAGCTCAAGGAGCTA
          10      20      30      40      50

          130     140     150     160     170     180
pCaBP  CTGACCAGGGAGCTGCCTAGCTTCTGGGGAAAAGGACAGATGAAGCTGCATTCAGAAAG
BG12   : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
          CTGACCAGGGAGCTGCCTAGCTTCTGGGGAAAAGGACAGATGAAGCTGCATTCAGAAAG
          60      70      80      90      100     110

          190     200     210     220     230     240
pCaBP  GTGATGAGCAACTTGGACAGCAACAGGGACAATGAAGTTGACTTCCAGGAGTACTGTGTC
BG12   : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
          GTGATGAGCAACTTGGACAGCAACAGGGACAATGAAGTTGACTTCCAGGAGTACTGTGTC
          120     130     140     150     160     170

          250     260     270     280     290
pCaBP  TTCCTGTCCCT-GCATTGCCATGATGTGCAATGAATTCCTTGGGGCTGCCAGATAAGGA
BG12   : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
          TTCCTGTCCCTTGCATTGCCATGATGTGCAATGAATTCCTTGGGGCTGCCAGATAAGGA
          180     190     200     210     220     230

          300     310     320     330     340     350
pCaBP  GCCCCGGAAGAAGTGAAGACTCCTCAGATGAAGTGTGGGGTGTAGTTGCCAGTGGGGG
BG12   : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
          GCCCCGGAAGAAGTGAAGACTCCTCAGATGAAGTGTGGGGTGTAGTTGCCAGTGGGGG
          240     250     260     270     280     290

          360     370     380     390     400     410
pCaBP  ATCTTCCCTGTTGGCTGTGAGCATAGTGCCTTACTCTGGCTTCTTCGCACATGTGCACAG
BG12   : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
          ATCTTCCCTGTTGGCTGTGAGCATAGTGCCTTACTCTGGCTTCTTCGCACATGTGCACAG
          300     310     320     330     340     350

          420     430     440     450
pCaBP  TGCTGAGCAAATTCATAAAAAGGTTTTGAAACT
BG12   : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
          TGCTGAGCAAATTCATAAAAAGGAAAAGAAACT
          360     370     380

```

Figure 3. Nucleotide sequence alignment of placental Ca^{2+} -binding protein (pCaBP; upper line), a MES-1 cDNA clone BG12 (lower line). Identical nucleotides are indicated (:).

```

      1570      1580      1590      1600      1610      1620
MMVIM CAGCTTTCAAGTGCCTTTACTGCAGTTTTTCAGGAGCGCAAGATAGATTTGGAATAGAAA
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
EH12   TTTTCAGGAGCGCAAGATAGATTTGGAATAGAAG
      10      20      30

      1630      1640      1650      1660      1670      1680
MMVIM GAAGCTCAGCACTTAACAACCTGACACCCCAAAAGACGTAGAAAAGGTTTACAAAATAATC
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
EH12   GAAGCTCAGCACTTAACAACCTGACACCCCAAAAGACGTAGAAAAGGTTTACAAAATAATC
      40      50      60      70      80      90

      1690      1700      1710      1720      1730
MMVIM TAG-TTTAGCAAGAAATCTTGTGCTAGAATACTTTTAAAGTATTTTTGAATACCATTAAA
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
EH12   TAGGTTTACGAAGAAACTCTGTGCTAGAATATTTTTAA-GTATTTTTGAATAC-ATTTAA
      100     110     120     130     140     150

      1750      1760      1770      1780      1790
MMVIM ACTGCTTTTTCCAGTAAATATCTGACCAACTTGTACTGCTTCAATAAATCTTCAA
      : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
EH12   ----CTGTTTTTCAGTAAATATCAGAC--ACTTGTTACTGCTTCA-TA----TCACA
      160     170     180     190

```

Figure 4

Nucleotide sequence alignment of mouse vimentin (MMVIM; upper line), and MES-1 cDNA clone EH12. Identical nucleotides are indicated (:).

database search did not result in the discovery of strong homologies among known gene sequences.

The identity of clone CA11 could be established after isolation of a full length cDNA. Comparison of the deduced amino acid sequence with known proteins revealed that the CA11 gene encodes cellular retinoic acid-binding protein (CRABP). Details of this experiment will be described in Chapter 3.

```

              70      80      90      100     110     120
RL-14 AACCTGAATCTCAAACCTGGGGAATGTCTCAAAGTTCGGGGAGAGCTGGCCCCGGACGCC
      FE11                                     : : : : : : : : : : : : : : : :
              10      20
      CGCTCGGGGAGAGGTGGCCTCGGACGCC

              130     140     150     160     170     180
RL-14 AAGAGCTTTGTGTTGAACCTGGGAAAGACAGCAACAACCTGTGCCTACACTTCAACCCC
      FE11 AAGACCTTTGTGCTGAACCTGGGAAAGACAGCAACAACCTGTGCCTACACTTCAATCCT
              30      40      50      60      70      80

              190     200     210     220     230     240
RL-14 CGCTTCAACGCCACGGAGATGCCAACACCATTGTGTGTAAACAGCAAGGACGATGGGACC
      FE11 CGCTTCAATGCCCATGGAGACGCCAACACCATTGTGTGTAAACAGCAAGGAAAGATGGGACC
              100     110     120     130     140

              250     260     270     280     290     300
RL-14 TGGGAAACAGAAACAACGGGAGACTGCCTTCCCTTCCAGCCTGGGAGCATCACGAGGTTG
      FE11 TGGGAAACGAAACACCGGAACTGCCTTCCCTTCCAGCCTGGGAGCATCACAGAGGTTG
              160     170     180     190     200

              310     320     330     340     350     360
RL-14 TGCATCACCTTTGACCAGGCTGACCTGACCATCAAGTGCCAGACGGGACATGAATTCAAA
      FE11 TGCATCACCTTTGACCAGGCTGACCTGACCATCAAGTGCCAGACGGGACATGAATTCAAAG
              220     230     240     250     260

              370     380     390     400     410     420
RL-14 TTCCCCAACCGCCTCAACATGGAGGCCATCAACTACATGGCGCGGATGGTACTTCAAG
      FE11 TTCCCCAACCGCCTCAACATGGAGGCCATCAACTACATGGCGCGGATGGAGACTTCAAG
              280     290     300     310     320

              430     440     450     460     470     480
RL-14 ATTAAGTGTGTGGCCTTTGAGTGAAGCCAGCCAGCCCCAGCCGTAGCCCCCAATAAAGG
      FE11 ATTAAGTGCCTGGCCTTTGAGTGAAGCCAGCCAGCC-----TGTAGCCCTCAATAAAGG
              340     350     360           370     380

              490     500     510
RL-14 CAGCTGCCTCTGCTCCCTGTAAACCAAAAAAAAAAAAA
      FE11 CAGCTGCCTCTGCTCGCCATATCCAGGTCTACAGTG
              390     400     410     420

```

Figure 5
Nucleotide sequence alignment of 14K rat β -galactoside binding protein (RL-14; upper line), and MES-1 cDNA clone FE11 (lower line). Identical nucleotides are indicated (:).

Chromosomal localisation of the murine β galactoside binding lectin (FE11) gene

The chromosomal assignment of the murine β galactoside binding lectin gene was determined using a panel of mouse/hamster somatic cell hybrids (Hilkens *et al.*, 1986). The FE11 cDNA insert was hybridized to filter blots containing EcoRI digested DNA isolated from the hybrid cell lines. Hybridization with the mouse-specific fragment was correlated with presence or absence of the mouse chromosome markers (see Table 3). 100% concordance was observed with chromosome 15 markers MYC and INT1, while ARS A scored 93.3%. We conclude that the murine β galactoside binding lectin gene is located on mouse chromosome 15.

Chromosomal localisation of the murine pCaBP (BG12) gene

Using the same panel of mouse-hamster somatic cell hybrids, (Hilkens *et al.*, 1986) the chromosomal localisation of the murine pCaBP gene was established. The murine cDNA probe BG12 readily detects a hybridizing fragment in Chinese hamster DNA, which is present in all hybrids. Hybridization with the mouse-specific fragment is observed only

```
1  AAGCGACAGC TGGCACGCGA AGAAGATCGG GTCACTAGTC AGTACGTACG
51  GGAGACCTAA GTCGGTAACC GGAAGAGCAC CGGTAGAAGG TGTTCATGAG
101 ACCGTTCTTT CCACTGTTTCG TGTGGGACTC GTTCTTCCTC GACTTCCTCA
151 CTAGGTCTTC CTCGAGTGGT AACCGAGGTT CGACGTCCTA CGACTTTAAC
201 GTTCCGACTA CCTACTAGAC CTGGCATTGT TCCTAGTCCT TCATTTGAAG
251 GTCCTCATAc AGCGGAAGGA CCCCCGGAAC CGAAACTAGA TGTTACTTTCG
301 AGACTTTATT TTACCATGGC AACTCTACTG AAGGCCCCCG GAGAGAGCCA
351 GTTTAGGTCA CCACCCATCA ATATGTATTA TAAAGCAAAA ACAATACGGA
401 AGAGAGCCAG TTTAGGTCAc CACCCATCAA TATGTATTAT AAAGCAAAAA
451 CAATACGGA
```

Figure 6
Nucleotide sequence of MES-1 cDNA clone EC2.

Marker	Chromosome	FE11 / chrom. marker				% concordance
		+/+	-/-	+/-	-/+	
PEP3	1	18	3	9	0	70.0
Bcl-2	1	16	3	7	0	73.0
SDH1	2	25	2	1	1	93.1
AK1	2	26	2	1	1	93.3
RASN	3	5	0	6	2	38.4
PGD	4	20	3	7	0	76.6
PGM2	4	17	3	10	0	66.6
ENO1	4	20	3	7	0	76.6
IF-	4	8	2	6	0	62.5
PEP-7	5	19	3	8	0	73.3
TPI1	6	16	0	11	3	53.3
LOC2	6	8	0	3	2	61.5
MET	6	16	0	10	2	57.1
IRP	6	11	1	7	2	57.1
GPI1	7	25	1	2	2	86.6
LDH1	7	25	1	2	2	86.6
PEP4	7	16	1	2	2	80.9
GR1	8	16	3	11	0	63.3
APRT	8	16	3	7	0	73.0
DIA	8	12	3	15	0	50.0
MOD1	9	18	3	9	0	70.0
MPI1	9	19	3	8	0	73.3
PK3	9	19	3	8	0	73.3
PEP2	10	20	2	7	1	73.3
BCR1	10	13	2	6	1	68.1
HK1	10	20	2	7	1	73.3
ESR	10	12	2	5	1	70.0
GALK	11	1	3	15	0	21.1
TCN2	11	0	2	14	0	12.5
PAD1	11	1	3	18	0	18.1
ACP1	12	24	0	3	2	82.7
ARSB	13	18	3	6	0	77.7
NP1	14	11	3	16	0	46.6
ES10	14	12	1	16	0	46.6
ARSA	15	27	1	0	2	93.3
MYC	15	16	2	0	0	100.0
INT1	15	8	2	0	0	100.0
SOD1	16	19	2	8	1	70.0
LOC3	16	7	1	4	1	61.5
SOD2	17	23	3	4	0	86.6
C3	17	11	3	4	0	77.7
PIM2	17	18	3	3	0	87.5
PEP1	18	17	3	6	0	76.9
GOT1	19	19	2	8	1	70.0
PB1	19	12	2	7	1	63.6

Table 3

Correlation between the presence or absence of the beta galactoside-binding lectin (FE11) gene and chromosome markers in mouse-Chinese hamster hybrid cells.

in hybrids that are positive for the chromosome 3 marker N-ras. Concordance scores for all other mouse chromosome markers were invariably lower. We conclude that the gene encoding pCaBP is located on mouse chromosome 3.

Cloning and chromosomal assignment of the human pCaBP gene

As can be seen in Figure 2, the murine pCaBP cDNA clone BG12 cross-hybridizes with human DNA. Single hybridizing fragments are obtained after digestion with different restriction enzymes, indicating that the BG12 probe recognizes a single copy gene in human. Screening of a human genomic library with the murine pCaBP cDNA probe resulted in the isolation of various overlapping bacteriophage clones. Restriction enzyme mapping demonstrated that the *EcoRI* site around position 275 (Jackson-Grusby *et al.*, 1987) is conserved between human and mouse. The repeat-free subclone P900 was derived from one of the BG12-positive human phages. The aptitude of P900 to serve as a human pCaBP probe was checked on Southern as well as on Northern blots, which resulted in hybridization to the same fragments resp. transcript as detected by the murine pCaBP probe BG12 (data not shown).

Using the P900 fragment as a probe for hybridization to DNAs extracted from human-hamster somatic cell hybrids (Geurts van Kessel *et al.*, 1983), a 100% concordance was observed between the presence or absence of chromosome 1 and pCaBP hybridization (see Table 5). High discordancy scores were obtained for all other human chromosomes. A refinement of this localisation was achieved with the aid of cell hybrids carrying fragments of chromosome 1 (Barneveld *et al.*, 1983; see Figure 7). From the latter results, we conclude that the human pCaBP maps to the region p32 to q31 of chromosome 1.

chromosome	pCaBP / chrom. marker				% concordance
	+/+	-/-	+/-	-/+	
*1	0	14	0	16	100
2	5	2	11	14	59
3	8	4	8	12	62
4	11	3	5	13	75
5	14	5	2	11	78
6	9	5	7	11	62
7	9	6	7	10	59
8	10	7	6	9	59
9	11	5	5	11	69
10	8	4	8	12	62
11	8	3	8	13	66
12	9	9	7	7	50
13	8	5	8	11	69
14	10	9	6	7	63
15	9	4	7	12	66
16	14	5	2	11	78
17	13	8	3	8	66
18	12	1	4	15	84
19	9	9	7	7	63
20	15	4	1	12	84
21	14	6	2	10	75
22	10	10	6	6	50
X	8	11	8	5	47

* two hybrids containing chromosome 1 translocation products were excluded from the

Table 5

Correlation between the presence or absence of the pCaBP gene and chromosome markers in human/chinese hamster hybrid cells.table.

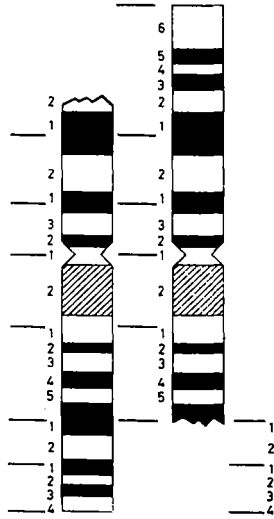


Figure 7

Translocation products used for regional mapping of pCaBP on human chromosome 1 (from Barneveld et al., 1983).

Discussion

In an attempt to identify genes that are developmentally regulated in the early differentiation of mammalian cells, we have isolated at least nine independent cDNA clones, corresponding to mRNAs that are highly expressed in the mesoderm-like P19 derived cell line MES-1, but not in undifferentiated P19 embryonal carcinoma cells. These cDNAs were obtained via differential screening of a MES-1 cDNA library. As only a limited number of 10.000 cDNA clones was screened, it is to be expected that the selected cDNA clones correspond to mRNAs that are relatively abundant in the MES-1 cell line. This abundance of transcripts in MES-1 cells, which was confirmed by Northern blot analysis, allowed us to perform comparative RNA analyses on total cellular RNA. However, by thus reducing sensitivity we cannot exclude that a particular cell line or tissue, now designated as negative for the presence of a probed mRNA, in fact expresses at low levels.

The P19 EC cell system was chosen for our experiments because of its ability to form derivatives of all three germ layers, which enabled us to determine whether the isolated gene sequences are specifically expressed during mesodermal differentiation. Alternatively, the observed expression could result from loss of the undifferentiated transformed phenotype. Three of the selected MES-1 cDNA clones (AA2, BG12, and CA11) apparently correspond to mRNAs specific for mesodermal differentiation of P19 EC.

As differentiation induction of P19 EC cells involves the use of chemical agents, it is important to determine whether the change in gene expression observed is indeed related to differentiation, or is an effect of the inducer as such. Expression studies using RNA isolated from C1003 EC and three of its mesodermal derivatives formed in response to temporary serum deprivation revealed that all but one of the selected MES-1 cDNA clones detect transcripts in the mesodermal derivatives but not in the parental cell line C1003. The observed mesoderm-specific expression in the C1003-derived cell lines excludes the possibility that the MES-1 cDNAs correspond to genes that were induced as a direct result of non-physiological treatment with DMSO. The one exception is clone CA11, which did not detect expression in C1003, nor in its mesodermal derivatives. However, this particular clone readily hybridizes to mouse embryonic RNA (see below), which suggests that expression of the CA11 gene can be related to cellular differentiation processes occurring *in vivo*.

Transcription of the genes corresponding to the selected MES-1 cDNA clones in mouse embryos and adult tissues was studied by Northern blot analysis. On the basis of the results obtained in these expression studies we selected clones AA2, BG12, EC2, CA11, EH12, and FE11 for further analysis. Clone EH12 detects transcripts in RNA isolated from all mouse organs, but expression in mouse embryos appears to be particularly high. The other four clones correspond to gene sequences that are expressed in a limited number of embryonic and adult tissues. Apart from EC2 and FE11, the selected clones show a mesoderm-specific expression pattern.

Southern blot analysis using the cDNA inserts of the selected clones as probes in hybridization revealed that clones BG12, CA11, EH12, FE11, and EC2 apparently correspond to single or a few copy genes, whereas clone AA2 evidently hybridizes to repetitive genomic sequences. For this reason, and also because this one clone did not cross-hybridize with DNA isolated from different species, AA2 was excluded from further analysis. Indeed, genes that have fundamental biological functions often show significant interspecies homology.

We have determined the nucleotide sequences of clones BG12, CA11, EH12, FE11, and EC2. In the case of BG12, CA11, EH12, and FE11, database comparison resulted in the identification of the corresponding genes. These results will be discussed for each clone separately.

Clone BG12: placental Ca²⁺-binding protein

The nucleotide sequence of clone BG12 was found to correspond to the cDNA sequence of placental Ca²⁺-binding protein (Jackson-Grusby *et al.*, 1987). This 9000-M_r protein,

which is identical to the vitamin D-dependent intestinal Ca^{2+} -binding protein or calbindin 10_k (McManus *et al.*, 1986), belongs to the troponin C superfamily of Ca^{2+} -binding proteins, which also includes calmodulin, troponin C and parvalbumin. Members of this multi-gene family are thought to be involved in the regulation of intracellular Ca^{2+} concentrations, thus mediating Ca^{2+} signal transduction. Proteins belonging to the superfamily of Ca^{2+} -binding proteins are characterized by the occurrence of one or more copies of a specific Ca^{2+} -binding structure, the so-called EF-hand motif (Heizmann and Berchtold, 1987). pCaBP belongs to a subclass of the troponin C family that contains two EF-hand motifs, and further comprises S100 α , S100 β , cystic fibrosis antigen, p11 protein, and the 2A9 protein (reviewed by Heizmann and Berchtold, 1987).

In agreement with a recent report by Dorin *et al.* (1990), we have localised the pCaBP gene, which contains two Ca^{2+} -binding motifs, on mouse chromosome 3 and human chromosome 1. An extensive synteny has been observed between mouse chromosome 3 and the peri-centromeric region of human chromosome 1 (see HGM 9, 1986). Our mapping data indicate that the pCaBP is yet another member of this synteny group. Interestingly, the gene encoding cystic fibrosis antigen has been assigned to the q12-q22 region of human chromosome 1 (Dorin *et al.*, 1987). The observed co-localisation of the genes for pCaBP and cystic fibrosis antigen on human chromosome 1 is in line with the idea that members of the troponin C gene family have evolved from a common one- or two-domained precursor gene via duplication and/or deletion mechanisms (Parmentier *et al.*, 1987).

Interestingly, the cDNA encoding pCaBP was also isolated by other research groups who performed differential screening of cDNA libraries from various sources. Jackson-Grusby *et al.* (1987) reported the characterization of an mRNA that increases in abundance after serum stimulation of quiescent mouse fibroblasts as encoding pCaBP. The identification of mRNAs which are abundant in established cell lines, but less so in the corresponding parental counterparts by Goto *et al.* (1988) also resulted in the isolation of the pCaBP cDNA. Comparison of a highly metastatic subline derived from a spontaneous mouse mammary carcinoma with a subline exhibiting a low metastatic potential showed that pCaBP is expressed specifically in metastatic cells (Ebraldize *et al.*, 1989). Thus, induction of pCaBP appears to play a role in various processes of cell differentiation, cell-cycle regulation, and cell proliferation.

Clone CA11: cellular retinoic acid binding protein

Clone CA11, which showed diminishing expression with increasing age of the embryo, is of special interest. The isolation of a full length CA11 cDNA and the subsequent

determination of the corresponding nucleotide and amino acid sequences, which led to the identification of CA11 as encoding the cellular retinoic acid binding protein (CRABP), will be discussed in Chapter III.

Clone EH12: vimentin

Determination of the nucleotide sequence of clone EH12, followed by database comparison, revealed that this clone corresponds to mouse vimentin. Vimentin is one of the intermediate filaments, and is typically found in cells of mesenchymal origin. It is also present in many cultured cells, including most EC cell lines and their differentiated derivatives (Paulin *et al.*, 1982). In this respect, EC cells are clearly different from early embryonic cells as during mouse embryonic development vimentin expression is not recognized prior to day 8 (Jackson *et al.*, 1981). Hence, it has been suggested that vimentin expression in EC cells is due to growth *in vitro* and is not related to the differentiation state of the cells. Our finding that the vimentin mRNA strongly increases in abundance during differentiation of P19 EC argues against this theory, and points to vimentin as an early marker of cellular differentiation.

Clone FE11: β galactoside binding lectin

We have been able to identify clone FE11 as encoding the mouse homolog of RL-14, the β galactoside binding lectin (Clerch *et al.*, 1988), and to map the corresponding gene on mouse chromosome 15. The 14 kD β galactoside binding lectin belongs to the family of soluble lectins, which appear to be secreted into the extracellular matrix, and have been implicated in cell-cell and cell-substratum adhesion (Barondes, 1984; Lotan and Raz, 1988). A large amount of RL-14 is synthesized during myoblast differentiation (Gartner and Podleski, 1975; Nowak *et al.*, 1976), which is consistent with the induced expression of the mouse homolog of the gene during mesodermal differentiation of P19 EC cells. However, high expression of RL-14 also occurs in the developing lung (Powell and Whitney, 1980), and the protein is found in adult lung, uterus, and heart as well (Powell, 1980). Thus, it is not surprising that we observed expression of the mouse homolog of RL-14 in cell lines representing the endodermal and ectodermal line of differentiation of P19 EC cells.

Clone EC2

The database search which was carried out with the EC2 cDNA sequence did not result in the discovery of any strong homologies. Apparently, EC2 does not correspond to any mRNA sequence contained within the databases of the European Molecular Biology Laboratory and the National Biomedical Research Foundation. The EC2 cDNA insert showed strong cross-hybridization with rat, hamster, and human DNA which suggests a strong evolutionary conservation of the gene. Therefore, it is likely that homology would have been detected even if the sequence in the database was derived from a different species. Thus, the failure to detect homologous sequences offers the interesting possibility that EC2 corresponds to a hitherto unknown gene. On the other hand, the EC2 cDNA inserts may simply be too short to warrant identification of the corresponding genes by database comparison. We have not been able to delineate an open reading frame within the EC2 cDNA sequences, suggesting that the larger part of the cDNA insert corresponds to the 3' untranslated region of the mRNA. Many protein sequences have been determined for which the corresponding genes have not been cloned. Therefore, isolation of a longer, if possible full-length, EC2 cDNA clone, followed by database comparison with the total cDNA sequence and the predicted amino acid sequence could still identify the corresponding gene or gene product.

In this study, we have shown that differential screening of a cDNA library, obtained from a teratocarcinoma derived cell line, has resulted in the isolation of cDNA clones corresponding to genes that are specifically expressed during EC cell differentiation. As most of the selected clones also hybridize to embryonic RNA, it is tempting to assume that induction of these genes also takes place during actual embryonic development. The identification of some of the corresponding genes products has yielded valuable clues to the biological function of the genes, but additional experiments are required to define their putative roles in embryogenesis.

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Chapter III

PREFERENTIAL EXPRESSION OF CELLULAR RETINOIC ACID-BINDING PROTEIN IN A SUBPOPULATION OF NEURAL CELLS IN THE DEVELOPING MOUSE EMBRYO.

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Differentiation 40, 99-105, 1990.

Abstract

The cellular retinoic acid binding protein is thought to be involved in the retinoic acid mediated signal transduction pathway. We have isolated the mouse cellular retinoic acid binding protein cDNA from an embryonal carcinoma derived cell line by using differential cDNA cloning strategies. *In situ* hybridization on sections of mouse embryos of various developmental stages indicated that the *cellular retinoic acid-binding protein* gene, which we localized on mouse chromosome 9, is preferentially expressed in a subpopulation of neurectodermal cells. This restricted expression pattern suggests an important role for cellular retinoic acid binding protein in murine neurogenesis.

Introduction

Ample evidence has been accumulated indicating that retinol (vitamin A) and its derivative retinoic acid (RA) play an important role in normal cellular differentiation and proliferation, and in the inhibition of growth of various malignant cells, including

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embryonal carcinoma (EC) cells (Strickland and Mahdavi, 1978; Lotan, 1980; Chytil, 1984). RA may also act as a teratogen in experimental animals (Kochar, 1967) and is known to be the cause of severe birth defects upon administration to pregnant women (Lammer *et al.*, 1985; Lott *et al.*, 1984). At present, the molecular mechanisms underlying RA action are the subject of intensive investigations. Two human retinoic acid receptors have recently been cloned and identified as belonging to the steroid/thyroid receptor family, members of which are known to act as inducible transcription enhancers (Giguère *et al.*, 1987; Petkovich, 1987; Brand *et al.*, 1988). It is generally believed that the complex of retinoic acid and retinoic acid receptor mediates the expression of specific genes. The discovery of Thaller and Eichele (1987) that RA forms a concentration gradient across the developing chick limb bud supports the idea that RA plays a role in pattern formation, affecting cell morphology through activation of specific sets of genes. Recently, Maden *et al.* (1988) showed that the cellular retinoic acid binding protein (CRABP) is also present in varying concentrations in the developing chick limb bud, forming a gradient reciprocating the RA gradient.

Another developmental effect of RA is the promotion of the differentiation of EC cells, which occurs in a concentration dependent way, and is known to be accompanied by increased transcription of various genes (Strickland and Mahdavi, 1978; Edwards and McBurney, 1983; Chytil, 1984). Since mouse EC cells share many characteristics with the pluripotent cells of the embryo itself (Martin, 1980), identification of genes which are transcriptionally regulated upon differentiation of EC cells may help to elucidate the molecular mechanisms involved in early mammalian development. Over the past few years, many research efforts have been aimed at the isolation of such genes (Wang *et al.*, 1985; Ikuma *et al.*, 1986). We have isolated several cDNA clones corresponding to mRNAs that are induced upon differentiation of pluripotent P19 EC cells by using the mesoderm-like clonal derivative MES-1 (Mummery *et al.*, 1986), and differential cDNA cloning strategies. The nucleotide sequence of one of these cDNA clones was determined, and identified as encoding mouse CRABP. Expression studies using Northern blotting and *in situ* hybridization techniques revealed that *CRABP* is preferentially expressed in a subpopulation of neuroectodermal cells in the developing mouse embryo. The CRABP-encoding gene was assigned to mouse chromosome 9.

Materials and Methods

Cell lines and culture techniques

P19 EC cells were cultured as described previously for PC13 (Mummery *et al.*, 1985). Mesodermal differentiation was induced as described by McBurney *et al.* (1982) by aggregating cells for 5 days in the presence of 1% dimethylsulfoxide (DMSO), then replating in tissue culture grade plastic dishes. After 7 days, the cultures consisted of a heterogenous mixture of fibroblast like cells with areas of rhythmically contracting muscle; no EC cells were evident. The MES-1 cell line was isolated by single cell picking from an area of contracting muscle from DMSO-treated aggregates (Mummery *et al.*, 1986).

The panel of mouse-Chinese hamster hybrid cells used for chromosomal localization of the *CRABP* gene has been described (Hilkens *et al.*, 1979, 1983, 1986). The cells were cultured according to standard procedures. DNA analysis and checks for the presence of chromosome specific markers were performed using the same batch of cells.

DNA isolation and blot hybridization

Total RNA was isolated from cell lines using the LiCl/Urea method described by Auffray and Rougeon (1980). The same procedure was applied for the isolation of RNA from BALB/c mouse embryos and adult tissues. For RNA blot analysis, RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde, and transferred to nitrocellulose filters (Maniatis *et al.*, 1982). DNA probes, labelled with ³²P using random priming as described by Feinberg and Vogelstein (1983) were hybridized to the RNA blots at 42°C in a buffer containing 9% dextran sulphate and 50% formamide. The filters were washed at 65°C, twice in 3xSSC, 0.1% NaDodSO₄ and twice in 1xSSC, 0.1% NaDodSO₄.

DNA isolation and blot hybridization

High molecular weight DNAs were extracted from cell lines according to standard procedures (Jeffreys and Flavell, 1977). Following cleavage with restriction enzymes, DNA fragments were separated by electrophoresis on agarose gels, and transferred onto nitrocellulose filters by Southern blot techniques (Southern, 1975). Hybridization of the DNA filters and washing conditions were essentially the same as described for RNA filters.

Construction and screening of the MES-1 cDNA library

Poly(A)⁺ RNA was purified from total cellular MES-1 RNA after two cycles of selection on an oligo(dT)-cellulose column (Aviv and Leder, 1972). cDNA synthesis was performed as described by Gubler and Hoffman (1983), using an oligo(dT) primer for reverse transcription of the RNA, followed by self-primed second strand synthesis. Double stranded cDNA was tailed with poly(dC), and annealed with *Pst*I-digested poly(dG) tailed pBR322 (Gibco BRL, Breda, The Netherlands). Transformation of *Escherichia coli* RR1 resulted in a library of about 5×10^4 clones. About 10^4 cDNA clones were cultured separately in 96 well plates, which were used as master plates, and replica-plated onto a pair of Genescreen-plus filters (NEN, Boston, Mass.). Colony hybridization (Grunstein and Hogness, 1975) was carried out using single stranded (³²P) cDNAs from poly(A)⁺ RNA of P19 EC and MES-1 cells.

Sequence analysis

The nucleotide sequence of mouse *CRABP* was obtained from the cDNA clone *MoT-CA11*, which is derived from a mouse testis cDNA library. This cDNA library consists of oligo(dT) primed cDNAs which were inserted into the *Eco*RI site of bacteriophage lambda gt10 through the use of adapters. For our nucleotide sequence analysis, fragments of *MoT-CA11* were subcloned into the plasmid vector pTZ18R or pTZ19R. The DNA sequence was determined via the dideoxy chain termination method (Sanger *et al.*, 1977), using Sequenase (United States Biochemical, Cleveland, Ohio) according to instructions issued by the manufacturer.

Computer-assisted analysis of the DNA sequence was carried out on a Vax 8530 using the Staden operating system. The National Biomedical Research Foundation Protein Sequence Database was searched with the predicted amino acid sequence of the *CA11* cDNA using the FASTP algorithm (Lipman and Pearson, 1985).

In situ hybridization techniques

Mouse embryos were embedded in Tissue Tek OCT, frozen, and sectioned at 8-10 μ m. Following attachment to chromium (III) potassium sulphate coated slides, the sections were fixed in 4% paraformaldehyde, dehydrated, and prepared for hybridization to ³⁵S-labelled DNA probes, nick-translated to a specific activity of $2-4 \times 10^8$ dpm/ μ g, and treated with DNase I to obtain fragments of approx. 50 - 100 bp. The *CRABP* probe used was a *Taq*I-*Taq*I fragment of *MoT-CA11*, containing nucleotides 148 to 315 (see Figure 2). Section pretreatment and *in situ* hybridization procedures were based on

standard methods (Akam, 1983; Hafen *et al.*, 1983). For autoradiography, slides were dipped in Kodak NBT-2 emulsion, and developed after 3-7 days of exposure. Counterstaining with haematoxylin was carried out as required. Sections were photographed using a Nikon microscope equipped with epipolarization optics.

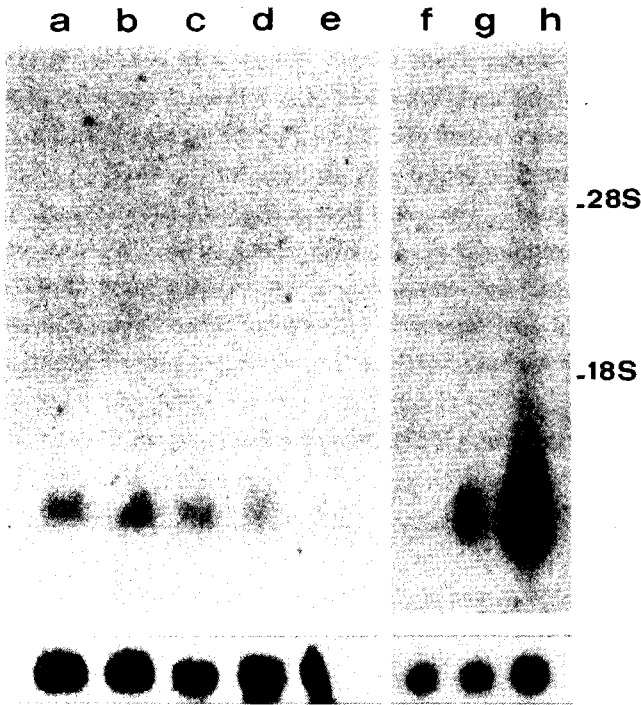


Figure 1

Hybridization of cDNA clone CA11 to RNA samples isolated from different sources. (a) day 9 mouse embryo, (b) day 11 mouse embryo, (c) day 13 mouse embryo, (d) day 16 mouse embryo, (e) day 19 mouse embryo, (f) P19 EC cells, (g) P19 EC cells treated with DMSO, and (h) MES-1 cells. The position of the ribosomal RNAs (28S and 18S) is indicated. Control hybridizations with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (a, b, c, d, e) or actin probes (f, g, h) are shown underneath. The ± 1 kb CA11 transcript is detected in all RNA samples except undifferentiated P19 EC cells.

Results

Isolation and identification of the mouse CRABP cDNA

Differentially hybridizing cDNA clones representing at least 13 different mRNAs were isolated from a MES-1 cDNA library (see Materials and Methods). Expression of these mRNAs was studied in various EC cell lines and mouse organs. Based on these expression studies, we selected the cDNA clone *CA11*, which corresponds to a transcript of ± 1 kb, for further analysis. The *CA11* gene is abundantly expressed in MES-1 as compared to undifferentiated P19 EC cells (Figure 1, lanes h and f). *CA11* transcription can readily be induced in P19 EC cells by treatment of cell aggregates with DMSO (Figure 1 lane g). The *CA11* transcript was also detected in poly(A)⁺ RNA isolated from adult mouse testis (not shown).

Screening of a mouse testis cDNA library with a *CA11* probe yielded clone *MoT-CA11*, which contains a cDNA insert of ± 800 bp. Fragments of *MoT-CA11* were subcloned into the plasmid vector pTZ18R or pTZ19R, and used for determination of the nucleotide sequence. The sequencing strategy for the *MoT-CA11* cDNA is outlined in the upper part of Figure 2. The lower part of Figure 2 shows the cDNA sequence and deduced amino acid sequence of the *CA11* gene. The cDNA is 760 bp in length, excluding the poly(A) tail, and contains an open reading frame of 408 bp. Comparison of the predicted amino acid sequence with known proteins through computer analysis revealed that our cDNA clone *MoT-CA11* codes for a protein identical to the bovine CRABP (Sundelin *et al.*, 1985). Comparison with the DNA sequence of bovine *CRABP* as published by Shubeita *et al.* (1987) showed a homology of 90% in the coding region.

Figure 2

Sequencing strategy, cDNA sequence, and deduced amino acid sequence of mouse CRABP. Determination of the nucleotide sequence of cDNA clone MoT-CA11 was carried out using the total EcoRI - EcoRI insert, and restriction fragments (EcoRI - TaqI, 160 bp; TaqI - TaqI, 170 bp; and TaqI - EcoRI, 490 bp) which were subcloned into the plasmid vectors pTZ18R or pTZ19R. Nucleotide residues are numbered according to the sequence of the cDNA clone MoT-CA11. The predicted amino acid sequence is given above the long open reading frame. Homology with the cDNA sequence of bovine CRABP as published by Shubeita et al. (1987) is shown on the third line, with asterisks indicating differing nucleotide residues. While the proteins encoded by mouse and bovine CRABP cDNA appear to be identical, homology at the DNA level is 90% in the coding region. A line is drawn above the putative polyadenylation signal, and a single dot indicates the termination codon. The nucleotides contained in cDNA clone CA11, which was initially isolated from a MES-1 cDNA library, are underlined; it contains only 3' untranslated sequences.

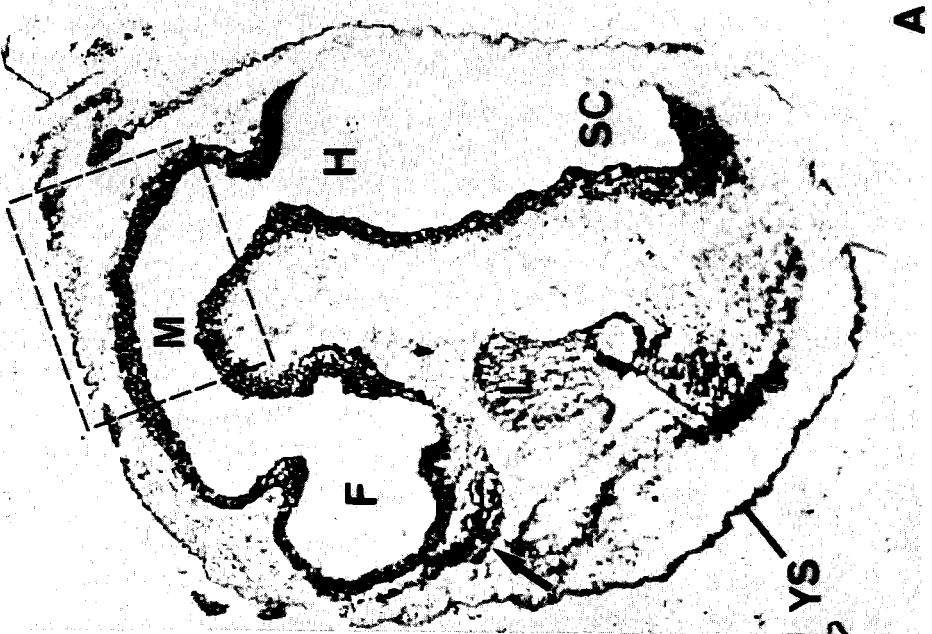
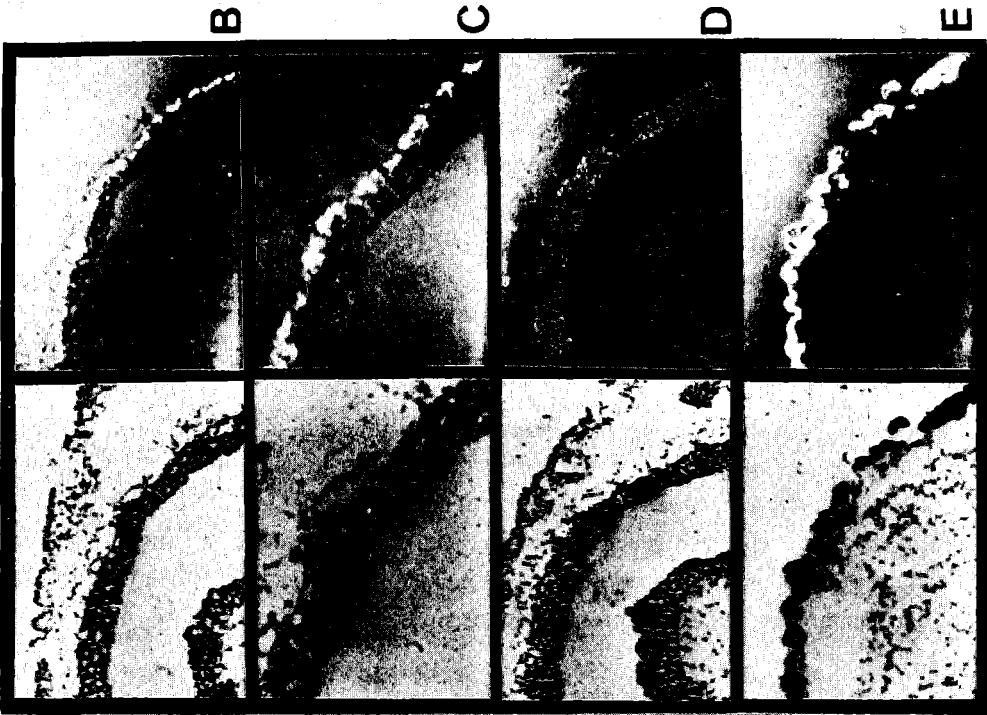
Expression of CRABP in the developing mouse embryo

Expression studies using the *CA11 (CRABP)* cDNA as a probe in hybridization to RNA blots revealed that the corresponding gene is preferentially expressed during embryogenesis. As shown in Figure 1, expression was observed in the earliest embryonal stage examined, which was at day 9 of gestation, and remained at about the same level until after day 16. A clear drop in expression was observed between days 16 and 19.

In order to determine the exact site of expression, we performed *in situ* hybridization experiments on serial sections of mouse embryos at various stages of development. Figure 3 shows a section through a day 9 mouse embryo, to which a probe, containing part of the coding sequence of mouse CRABP, was hybridized. From this section, and a series of longitudinal and transversal sections taken from the same and other embryos and hybridized with a CRABP probe, we conclude that a high level of CRABP mRNA is present in a subpopulation of neural cells. The cells that show a strong CRABP specific hybridization signal are located in the outer layer of the neural tissue. They are observed in the dorsal part of the midbrain (mesencephalon) and the anterior part of the hindbrain (rhombencephalon). More posterior in the rhombencephalon, a gradual transition of the CRABP specific hybridization signal from lateral to ventro-lateral occurs, and continues into the spinal cord. No hybridization of the CRABP

Figure 3

Localization of CRABP transcripts in a day 9 mouse embryo. Figure 3A shows an entire longitudinal section, in which the following structures can be recognized: forebrain (diencephalon) (F), midbrain (mesencephalon) (M), hindbrain (rhombencephalon) (H), spinal cord (SC), liver (L), and yolk sac (YS). A dark, CRABP specific hybridization signal is present in the outer dorsal cell layer of the midbrain and the anterior part of the hindbrain, in the outer ventral cell layer of the posterior part of the hindbrain, and the spinal cord. Expression of CRABP is also detected in a group of mesenchymal cells, located anterior to the forebrain (indicated by an arrow). In Figure 3B, part of the same section as shown in 3A (boxed area) is photographed using bright field (left) and epipolarization (right) optics, which clearly shows the CRABP specific labelling in the roof of the mesencephalon. Figure 3C gives a detail of the group of mesenchymal cells that show high CRABP expression, photographed as indicated for fig. 3B. Figures 3D and 3E show control hybridizations on sections from the same embryo as in Figure 3A, B and C. Figure 3D should be compared with 3B, showing part of the midbrain in an adjacent section, to which a GAPDH probe was hybridized. Specific labelling occurs in all cells present in the section, as becomes apparent using epipolarization optics (right). In figure 3E, expression of AFP in the yolk sac is visualized, again photographed as indicated for fig. 3B. AFP specific labelling is restricted to the cells of the yolk sac.



probe is observed in the forebrain (diencephalon), the auditory vesicle, and the caudal part of the spinal cord. Apart from the central nervous system, a high level of *CRABP* transcripts was also detected in a group of mesenchymal cells located anterior to the forebrain, and in cells of the transversal septum of the developing liver.

Figure 3B-E gives details of a number of sections, taken from the same day 9 mouse embryo, showing hybridization with our *CRABP* probe (Figure 3B, C and D), and a control alpha fetoprotein (*AFP*) (3E) probe. In Figure 3B and 3C, which was taken at a higher magnification, the *CRABP* specific hybridization signal in the outer dorsal cell layer of the midbrain is clearly demonstrated. Figure 3D gives a detail of the group of mesenchymal cells located anterior to the forebrain that show a high *CRABP* expression. As expected, *AFP* transcripts are detected only in the cells of the visceral yolk sac as is illustrated by Figure 3E. *CRABP* expression was also detected in sections obtained from day 11 mouse embryos. Again, the level of expression was elevated in the outer cell layers of the central nervous system, but not in telencephalon and diencephalon. A significant level of *CRABP* specific hybridization was observed in all the cells of the central nervous system, indicating a less restricted expression pattern as compared to day 9. In day 13 embryos, *CRABP* expression appeared to be even more diffuse (not shown).

Chromosomal localization of the mouse CRABP gene

A panel of 30 Chinese hamster-mouse somatic cell hybrids was used for the chromosomal localization of the *CRABP* gene. These hybrid cells had retained complete sets of Chinese hamster chromosomes and only limited numbers of mouse chromosomes. High molecular weight DNA extracted from these cells and control hamster and mouse cells was cleaved with *EcoRI* and, after Southern blotting, analyzed for the presence of *CRABP* specific sequences using the *CA11* cDNA as probe. This probe hybridizes to clearly distinct *EcoRI* fragments in mouse and hamster DNA, respectively (Figure 4, lanes d and e). The presence or absence of the murine fragment in the hybrid cells was correlated with the presence or absence of mouse chromosome specific markers as shown in Table 1. A 100% concordance was observed with the chromosome 9 markers MPI1 and PK3, and a 96.1% concordance with the chromosome 9 marker MOD1. For all other murine chromosome markers concordance scores were invariably lower, which allows the unambiguous assignment of the *CRABP* gene to mouse chromosome 9.

Marker	Chromosome	CRABP / chrom. marker				% concordance
		+/+	-/-	+/-	-/+	
PEP3	1	13	8	3	2	80.7
Bcl-2	1	12	7	2	3	79.1
SDH1	2	15	3	0	7	72.0
AK1	2	16	3	0	7	73.0
RASN	3	3	4	2	3	58.3
PGD	4	12	5	4	5	65.3
PGM2	4	12	8	4	2	76.9
ENO1	4	12	5	4	5	65.3
IF-	4	5	5	2	1	76.9
PEP-7	5	12	5	4	5	65.3
TPI1	6	9	3	7	7	46.1
LOC2	6	5	0	3	5	38.4
HB	6	6	3	5	7	42.8
GPI1	7	15	2	1	8	65.3
LDH1	7	15	2	1	8	65.3
PEP4	7	11	2	1	4	72.2
GR1	8	11	7	5	3	69.2
APRT	8	11	5	4	3	69.5
DIA	8	7	7	9	3	53.8
MOD1	9	15	10	1	0	96.1
MPI1	9	16	10	0	0	100.0
PK3	9	16	10	0	0	100.0
PEP2	10	15	7	1	3	84.6
BCR1	10	10	7	1	3	80.9
HK1	10	15	7	1	3	84.6
ESR	10	9	6	1	3	78.9
GALK	11	2	6	8	0	50.0
TCN2	11	0	5	9	0	35.7
SHU	11	0	9	7	1	52.9
MUB1	11	0	5	5	0	50.0
PAD1	11	0	7	9	1	41.1
ACP1	12	16	2	0	7	72.0
ARSB	13	11	7	2	3	78.2
NP1	14	5	7	11	3	46.1
ES10	14	6	7	10	3	50.0
ARSA	15	16	1	0	9	65.3
MYC	15	10	2	0	4	75.0
SOD1	16	12	5	4	5	65.3
LOC3	16	6	3	2	2	69.2
SOD2	17	16	7	0	3	88.4
C3	17	7	6	0	4	76.4
PIM2	17	13	5	0	4	81.8
PEP1	18	12	6	3	2	78.2
GOT1	19	12	6	4	4	69.2
PB1	19	8	6	3	4	66.6

Table 1

Correlation between the presence or absence of the CRABP gene and chromosome markers in mouse-Chinese hamster hybrid cells.

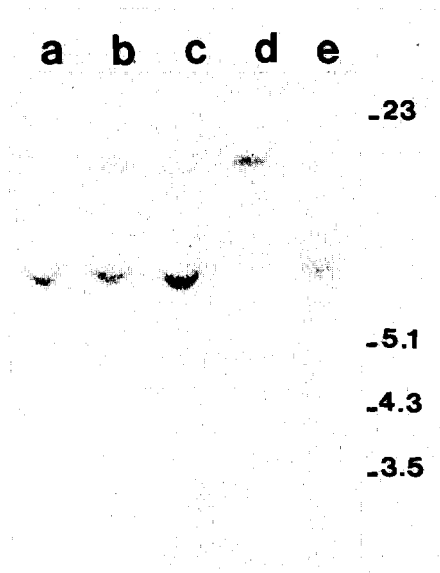


Figure 4

Southern blot analysis of EcoRI digested hybrid (lanes a,b,c) and parental (lanes d,e) cell DNAs (ca. 10 [g per lane) for the presence of CRABP sequences. Lane a: mouse CRABP negative, and lanes b and c: mouse CRABP positive hybrids, respectively. Lane d: mouse, and lane e: Chinese hamster CRABP hybridizing fragments. The indicated molecular weight markers (in kilobases) correspond to co-electrophoresed HindIII - EcoRI digested bacteriophage λ DNA.

Discussion

Via differential screening of a MES-1 cDNA library we have isolated a cDNA clone encoding the mouse CRABP. The corresponding gene is located on mouse chromosome 9, and its expression in the developing mouse embryo occurs in a spatio-temporally specific way.

Our results indicate that high CRABP expression in the mouse occurs around the stage at which development of somites and neuromeres is initiated (day 8-9) (Rugh, 1968). This expression continues till day 16 of gestation and diminishes during subsequent stages of embryonic development. At day 9, the elevated CRABP expression is restricted to a subpopulation of neural cells in the developing central nervous system, a distinct group of mesenchymal cells located anterior to the forebrain, and the transverse septum of the developing liver. At later stages expression appears to be more

diffuse. The observed high *CRABP* expression in a subpopulation of neural cells and in a limited number of mesenchymal cells during murine embryogenesis may represent a regulatory function. The recent finding that RA serves as a morphogen in normal embryogenesis (Thaller and Eichele, 1987), together with its teratogenic effects, seem to imply a role for RA in determining growth and differentiation of certain cells and tissues. The RA binding protein appears to be involved in the generation of RA concentration gradients across the developing chick limb bud (Maden *et al.*, 1988). Our expression data are consistent with this theory, pointing to CRABP as a putative mediator in the establishment of *in vivo* RA concentration gradients in the developing central nervous system. This suggestion is strengthened by a recent observation showing preferential accumulation of radioactively labelled RA in the neuroepithelium and developing central nervous system in day 10 and 11 mouse embryos, following intravenous injection of pregnant mice on day 9 *post coitum* (Dencker *et al.*, 1987). In analogy to its role in chicken limb bud development, CRABP-mediated RA concentration gradients may also affect regional differentiation within the central nervous system. The 100% conservation of CRABP at the amino acid level, observed between cow and mouse (estimated evolutionary separation 50 - 100 million years (Baba *et al.*, 1981) is completely in line with such an important role.

The assignment of the *CRABP* gene to mouse chromosome 9 is in agreement with, and further refines the provisional localization reported by Wei *et al.* (1987) using a bovine probe on a limited number of hamster-mouse somatic cell hybrids. Interestingly, two cellular retinol binding protein genes (*CRBP* and *CRBP II*) also map on mouse chromosome 9 (Demmer *et al.*, 1987). This clustering of retinoid binding protein genes on one chromosome and their low but significant sequence homology suggests that these genes may have evolved from a common ancestor gene. Several developmental neural and neuromuscular disorders have been assigned to mouse chromosome 9 (see e.g. Mouse News Letter, 1988), but a possible interrelationship with retinoid binding proteins awaits further investigation.

In our studies we have found that a mouse cDNA clone, selected on the basis of its ability to detect an abundant transcript in a mesoderm-like derivative of P19 EC but not in total RNA of the undifferentiated EC cell line, corresponds to mouse *CRABP*. The observed expression of *CRABP in vivo* in neuroepithelial tissues does not seem to correlate with its expression in a cell line exhibiting primarily mesodermal characteristics. The high level of expression found in a distinct group of mesenchymal cells, however, raises the possibility that MES-1 may be a representative of this particular cell type. The observed induction of *CRABP* expression as a result of DMSO treatment of P19 EC cells awaits further explanation but could be useful in clarifying molecular mechanisms underlying this particular line of differentiation. The isolation of a *CRABP* cDNA clone

from a MES-1 cDNA library is a typical example of how, through differential cDNA cloning strategies, gene sequences that play an important role in embryogenesis and possibly also in tumor development can be identified.

Acknowledgements

The authors thank S.W. de Laat for critical reading of the manuscript, and D. Meijer for gift of the testis cDNA library used. We also acknowledge O.H.J. Destrée, K. Lawson, J.H.C. Meijers, M. Mulder, T. Splinter, A.J. van Agthoven and J. van Baal for advice and support. We thank Mrs. R. Boucke for typing the manuscript, and P. Visser and C. van der Wiel for help with the preparation of the figures. This work was supported by the Netherlands Cancer Society (Koningin Wilhelmina Fonds).

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Chapter IV

THE CELLULAR RETINOIC ACID-BINDING PROTEIN IS EXPRESSED IN TISSUES ASSOCIATED WITH RETINOIC-ACID-INDUCED MALFORMATIONS

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Development 110, 371-378, 1990.

Abstract

Retinoic acid (RA) is thought to play a role in embryonic pattern formation in vertebrates. A naturally occurring gradient of endogenous RA has been demonstrated in the developing chick limb bud, while local application of RA leads to the formation of additional digits. In mammals, a well-defined spectrum of birth defects has been reported as a result of fetal exposure to excess RA. In analogy to the chick limb bud, it may be speculated that these malformations are the result of disturbance of morphogenetic RA concentration gradients.

A candidate gene involved in the regulation of endogenous RA concentrations is the gene encoding cellular RA binding protein (CRABP). We have isolated a partial cDNA clone corresponding to the chicken homolog of CRABP, and performed *in situ* hybridization experiments on sections of embryos at various stages of development. CRABP expression was detected in the CNS, the craniofacial mesenchyme, ganglia of the peripheral nervous system, the limb bud, and the visceral arch area. Our results indicate that the spatio temporally specified expression pattern displayed by the CRABP gene exhibits a striking correspondence to the tissues that are affected by exposure of avian or mammalian embryos to RA. We hypothesize that CRABP plays an important role in

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normal embryogenesis and that embryonic tissues showing high CRABP expression are susceptible to the adverse effects of excess RA.

Introduction

All-*trans* retinoic acid (RA), a biologically active metabolite of retinol (vitamin A), plays an important role in cellular differentiation and pattern formation during embryonic development of vertebrate animals. In chicken embryos, RA was found to be present in physiological concentrations similar to those known to be sufficient for the induction of EC cell differentiation *in vitro* (Thaller and Eichele, 1987). In the same study, Thaller and Eichele also demonstrated that RA is present in a higher concentration in the posterior part of the developing limb bud as compared to the anterior part. Local application of RA to the anterior margin of the limb bud results in dose-dependent changes in the skeletal pattern (Tickle *et al.*, 1982; Eichele, 1989; Tickle *et al.*, 1989). Local application of RA to a chick blastoderm grown *in vitro* was shown to interfere with axis formation (Mitrani and Shimoni, 1989). In *Xenopus laevis*, RA was found to act on differentiation of the central nervous system (CNS), causing transformation of anterior neural tissue to a posterior neural specification (Durstun *et al.*, 1989). Thus, the conclusion is warranted that RA acts as a morphogen in various developmental processes.

RA has long been known to be teratogenic in humans (Kochar, 1967), and has been reported to be the cause of severe birth defects when administered to pregnant women (Lammer *et al.*, 1985). RA-induced malformations include defects of the CNS (mainly hydrocephalus), cleft lip and cleft palate, and congenital heart defects. In rodents, the same spectrum of birth defects was observed, while high doses of RA have also been reported to result in limb deformations (Kochar 1973). Also in chicken embryos, the tissues affected by RA treatment seem to be similar to those reported for mammals (Jelinek and Kistler, 1981). Excess RA obviously disturbs differentiation of the CNS in *Xenopus laevis* (Durstun *et al.*, 1989). Altogether, it seems likely that RA-induced malformations represent a phenomenon general to all vertebrates.

If RA gradients influence morphogenetic processes during embryonic development, it is important to know which mechanisms regulate RA concentrations. In addition to regulation of the synthesis and degradation of RA, fine-tuning of RA levels may be achieved at the cellular level. In this respect, a possible role could be reserved for the cellular RA-binding protein CRABP. This 15.5×10^3 M_r protein, which displays a highly specific binding affinity for RA (Ong and Chytil, 1975; Jetten and Jetten, 1979), shows a structural similarity to the P2 family of proteins (Eriksson *et al.*, 1981; Sundelin

et al., 1985). Members of this family, which also includes cellular retinol binding protein (CRBP), are small, cytoplasmic proteins that have been implicated in the transport of specific small hydrophobic molecules.

CRABP exhibits a spatio temporally restricted expression pattern in the mouse embryo (Vaessen *et al.*, 1989a). Using *in situ* hybridization techniques, we detected a high level of CRABP transcripts in a subpopulation of cells in the CNS, as well as in the craniofacial mesenchyme. These results were later confirmed by others (Perez-Castro *et al.*, 1989). It was also shown that CRABP is differentially expressed in the developing limbs of the mouse (Perez-Castro *et al.*, 1989; Dollé *et al.*, 1989). Maden *et al.* (1989) reported on the immunocytochemical localization of CRABP in the chicken embryo. Apart from particular cells of the neural tube, CRABP was also found in various neural crest derivatives, including dorsal root ganglia and enteric ganglia. Earlier, these authors showed that a gradient of CRABP is present at the tip of the chick limb bud, with its maximum concentration in the anterior part (Maden *et al.*, 1988).

Apart from the limb bud, all major CRABP expression sites reported are of neuroectodermal origin, and notably include many neural crest derivatives. Because of this observation, and also because neural crest cells have been mentioned as candidate targets for RA-induced malformations (Poswillo, 1975; Lammer *et al.*, 1985), it would be of interest to know whether other neural crest cell derivatives also show high levels of CRABP expression. In order to study CRABP expression during embryogenesis, we performed *in situ* hybridization experiments on chicken embryos at various stages of development. Use of the chicken embryo for expression studies facilitated the acquisition of well-standardized material of early developmental stages. Furthermore, neural crest cell migration has been most thoroughly studied in avian embryos, and several markers for early neural crest cells have become available (reviewed by Anderson, 1989). We chose to use the monoclonal antibody HNK-1 (Abo and Balch, 1981), which recognizes a sulfated glucuronic acid present on several cell adhesion molecules (Kruse *et al.*, 1984,1986; Pesheva *et al.*, 1987; Rathjen *et al.*, 1987; Hoffman and Edelman, 1988). In the chicken embryo HNK-1 binds to most premigratory neural crest cells and neuronal neural crest cell derivatives (Vincent *et al.*, 1983; Vincent and Thiery, 1984; Tucker *et al.*, 1984). In addition, HNK-1 is an early marker for neural differentiation induced by RA in murine embryonal carcinoma cells (McBurney *et al.*, 1988).

Our results show that the CRABP gene exhibits a spatio temporally specified expression pattern, which offers a striking correlation to parts of the embryo that correspond to the tissues commonly affected by RA-induced malformations. The observed correlation is highly suggestive for a role of CRABP in RA-mediated morphogenetic processes, and may help to understand the underlying molecular mechanisms.

Materials and Methods

Isolation of a chicken CRABP cDNA clone

A chicken embryo cDNA library, consisting of oligo(dT)-primed cDNAs inserted into the *EcoRI* site of bacteriophage lambda gt11 (Clontech), was screened with a mouse CRABP cDNA probe. This probe, a 170 bp *TaqI-TaqI* fragment isolated from clone MoT-CA11 and containing part of the CRABP-coding region (Vaessen *et al.*, 1989^a), was labelled with ³²P using random priming (Feinberg and Vogelstein, 1983). The filters were hybridized at 56°C, in a buffer containing 6x SSC and 9% dextran sulphate, and washed at the same temperature, twice in 3x SSC, 0.1% NaDodSO₄, and twice in 1x SSC, 0.1% NaDodSO₄.

Screening of approximately 1x10⁶ bacteriophage plaques resulted in the isolation of a cDNA clone, designated C4. DNA isolated from clone C4 was digested with *EcoRI*, and ligated to *EcoRI* plasmid vector pTZ18R (Pharmacia). Transformation of *E. coli* DH5αF' yielded the subclone ChCRABP C4.5, which contains a cDNA insert of approximately 300 bp.

Sequence analysis.

For determination of the nucleotide sequence, the ChCRABP cDNA insert was subcloned into bacteriophage M13mp19. The cDNA insert was cloned in both orientations, allowing both strands to be read. After isolation of single-stranded DNA, the nucleotide sequence was determined via the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase (United States Biochemical) according to instructions by the manufacturer.

Computer-assisted analysis of the DNA sequence was carried out with a Microgenie software package (Beckman).

Chicken embryos

Fertilized eggs from the White Leghorn (*Gallus domesticus*) were incubated at 38°C in a forced-draft incubator at a relative humidity of 80%. Staging of the embryos was performed according to Hamburger and Hamilton (1951).

RNA isolation and blot hybridization

Total RNA was isolated from whole chicken embryos using the LiCl/Urea method described by Auffray and Rougeon (1980). For RNA blot analysis, RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde. Prior to electrophoresis, ethidium bromide was added to the RNA samples in order to allow visualisation of the ribosomal RNAs in the gel. In this way, it was ascertained that the amounts of RNA in the different lanes were approximately the same.

Following electrophoresis, the RNA was transferred to nitrocellulose filters (Maniatis *et al.*, 1982). DNA probes were labelled as described above. The RNA blots were hybridized at 42°C in a buffer containing 6x SSC, 9% dextran sulphate, and 50% formamide, and washed at 56°C, twice in 3x SSC, 0.1% NaDodSO₄, and twice in 1x SSC, 0.1% NaDodSO₄.

Preparation of embryo sections

Chicken embryos were fixed for 24 h in 4% paraformaldehyde in phosphate-buffered saline at 4°C. After fixation, the embryos were embedded in paraffin. Sections were cut at 5 µm, placed on chromium(III)potassium sulphate coated slides, and air dried.

Prior to *in situ* hybridization or immunocytochemical treatment, the sections were deparaffinized and hydrated.

In situ hybridization techniques

We used the cDNA insert of ChCRABP C4.5, labelled with ³⁵S via nick translation, and treated with DNaseI to obtain fragments of approximately 50-100 bp as a probe for *in situ* hybridization to the chicken embryo sections.

Section pretreatment and *in situ* hybridization procedures were based on standard methods (Akam, 1983; Hafen *et al.*, 1983). For autoradiography, slides were dipped in Kodak NBT-2 emulsion, and developed after 5-10 days of exposure. Counterstaining with haematoxylin was carried out as required.

Immunocytochemistry

The HNK-1 hybridoma cell line was purchased from the American Tissue Culture Collection (ATCC TIB 200). HNK-1 immunoperoxidase staining was performed using undiluted supernatant. Rabbit anti-mouse peroxidase-conjugated immunoglobulins (Dako, Denmark) were used in a dilution of 1:100. In order to reduce background staining, 2%

chick serum was added to the conjugate. Peroxidase was visualized by 0.1% 3,3'-diaminobenzidine.4HCl (Serva) and 0.02% hydrogenperoxide. All rinsing and diluting was done in phosphate-buffered saline with 0.1% Tween 20.

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EcoRI G S E N F D E L L K A L G V N A M
GAATTCGGCAGCGAGAATTCGACGAGCTCCTCAAAGCGCTGGGTGTCACGCCAATG
      * * * * *
CAGCAGCGAGAATTCGACGAGCTCCTCAAAGCGCTGGGTGTCACGCCAATG
S S E N F D E L L K A L G V N A M

L R K V A V A A A S K P H V E I R Q D G
CTCAGGAAGGTGGCGGTGGCGGCCCTCCAACCCACGTGGAGATCCGCCAGGACGGG
      * * * * *
CTGAGGAAGGTGGCGGTGGCGGTGCGTCTAAGCCGACGTGGAGATCCGCCAAGACGGG
L R K V A V A A A S K P H V E I R Q D G

D Q F Y I K T S T T V R T T E I N F K I
GACCAGTTCTACATCAAACCTCCACCACTGTCCGCACCACGGAAATCAACTTCAAATC
      * * * * *
GATCAGTTCTACATCAAGACATCCA TACTGTGCGCACCACGGAGATCAACTTCAAGTTC
D Q F Y I K T S T T V R T T E I N F K V

G E S F E E E T V D G R K C R S L A T W
GGGAGAGCTTCGAGGAGGAGACGGTGGATGGCCGAAATGCGAGGATTTGGCCACCTGG
      * * * * *
GGAGAGGGCTTCGAGGAGGAGACAGTGGACGGACGCAAAATGCGAGGATTTACCCACGTGG
G E G F E E E T V D G R K C R S L P T W

E N E N K I Y C K Q T L I E G D G P K T
GAGAATGAAAACAAGATCTATTGCAAACTCTTATTGAGGGAGATGGTCCATAAACA
      * * * * *
GAGAATGAGAACAAGATTCAGTGCACACAGACACTTCTTGAGGGGATGGCCATAAAT
E N E N K I H C T Q T L L E G D G P K T

Y W T EcoRI
TACTGGACTCGAATTC
      *
TACTGGACCC
Y W T

```

Figure 1
Sequence of the partial cDNA clone encoding chicken CRABP. The cDNA sequence and deduced amino acid sequence of ChCRABP C4.5 are shown in bold face, with underneath the corresponding sequence of mouse CRABP cDNA clone MoT-CA11. Non-homologous nucleotides are indicated by asterisks, and non-homologous amino acids are underlined. The EcoRI sites that flank the C4.5 cDNA insert are indicated.

Results

Isolation of a chicken CRABP cDNA clone

We obtained a chicken CRABP probe by screening a chicken embryo cDNA library with a *TaqI* - *TaqI* fragment containing nucleotides 147 - 315 of mouse CRABP cDNA clone MoT-CA11. This resulted in the isolation of bacteriophage clone C4, from which an *EcoRI* subclone in plasmid vector pTZ18R was derived. This subclone, which was named ChCRABP C4.5, contains a 310 bp cDNA insert, which corresponds to nucleotides 129 - 438 of mouse CRABP clone MoT-CA11. The sequence of ChCRABP C4.5 and its comparison with the mouse CRABP sequence is shown in Figure 1. The chicken cDNA clone contains part of the CRABP-coding sequence. Homology with mouse CRABP is 87% at the nucleotide level and 94% at the amino acid level.

The ³²P-labelled ChCRABP C4.5 insert DNA was hybridized to nitrocellulose blots containing total RNA isolated from chicken embryos at various stages of development. This resulted in detection of one single transcript of approximately 1 kb (see Figure 2), which is in agreement with the size of the mouse CRABP mRNA (Vaessen *et al.*, 1989a). Thus, ChCRABP C4.5 specifically hybridizes to chicken CRABP mRNA and can be used to study CRABP expression patterns in the chick embryo.

Localization of CRABP transcripts in chicken embryos

We performed *in situ* hybridization experiments with the radiolabelled cDNA insert of ChCRABP C4.5 on sections obtained from chicken embryos at various stages of development. The CRABP gene exhibits a strongly restricted expression pattern in day 2, day 3 and day 4 embryos, which tends to become more diffuse from day 5 onwards. High CRABP expression was found in the CNS, in the craniofacial mesenchyme, in the visceral arches, and in the ganglia of the peripheral nervous system. CRABP transcripts were also detected in the limb bud, where expression was predominantly found in mesenchymal cells located in the anterior part. In order to establish a possible relationship between expression sites of CRABP and the occurrence of migratory neural crest cells, we performed immunostaining experiments with the neural crest cell marker HNK-1 on sections serial to those used for the *in situ* hybridizations.

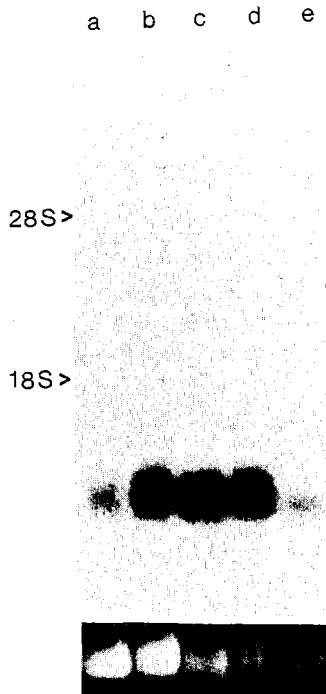


Figure 2

Hybridization of ChCRABP C4.5 to RNA samples isolated from (a) day 2; (b) day 3; (c) day 4; (d) day 5; and (e) day 6 chicken embryos. The position of the 28S and 18S ribosomal RNAs is indicated. The 18S ribosomal RNAs, stained with ethidium bromide, are shown underneath the corresponding lanes.

The following results were obtained:

(A). CRABP expression in stage 12 embryos

Figures 3 and 4 show serial sections of a stage 12 embryo (day 2) that were subjected to *in situ* hybridization with a CRABP probe (Figure 3) and immunostaining with HNK-1 (Figure 4). Occasional CRABP-positive cells were observed in the outer cell layer of the mesencephalon, rhombencephalon and spinal cord, but not in the prosencephalon. In the cells of the auditory pit, which is deep and wide open at this stage, CRABP transcripts were also detected. These cells exhibit strong expression of the HNK-1 epitope, while the cells of the neural tube are negative. CRABP expression was also observed in the

craniofacial mesenchyme anterior to the telencephalon and diencephalon. These mesenchymal cells are also positive for HNK-1.

(B). CRABP expression in stage 22 embryos

In stage 22 embryos (day 3), cells that show a strong hybridization to the CRABP probe were detected in the outer cell layers of mesencephalon, rhombencephalon and spinal cord. CRABP-positive cells are lying apart or in small clusters, but are more abundant than in stage 12 embryos (see Figure 5). The distribution of CRABP transcripts coincides with HNK-1 expression, as illustrated by Figure 6. As can be seen in Figure 7, CRABP expression is also observed in the neuroepithelial cells of the auditory vesicle, which is HNK-1 positive. The craniofacial mesenchyme, located anterior to telencephalon and diencephalon, and posteriorly confined by the eyes, exhibits a high level of CRABP transcripts (see Figure 5). These cells no longer express the HNK-1 epitope at this stage.

In addition to the CNS and the craniofacial mesenchyme, CRABP-positive cells were detected in the visceral arches, as illustrated by Figures 7 and 8. The CRABP-positive region extends from the craniofacial mesenchyme of the head into the first or mandibular arch, where an even higher level of CRABP transcripts was observed. High CRABP expression continues into the second, third and fourth arch. The hybridization signal appears to be strongest in the mesenchymal cells bordering the overlying epithelium of pharynx, visceral clefts, and pericardial cavity, while the epithelium itself is CRABP negative. In the visceral arch area, only occasional HNK-1 positive cells were observed.

CRABP expression was also detected in cranial and dorsal root ganglia, which are positive for HNK-1.

(C). CRABP expression in stage 24 embryos

In stage 24 embryos (day 4), a high level of CRABP expression was observed in the dorsolateral part of mesencephalon and metencephalon, extending to lateral and ventral, and continuing into myelencephalon and spinal cord. CRABP-positive cells were also observed in the ventral part of telencephalon and diencephalon, posteriorly confined by the olfactory pit, which is CRABP negative. The strong CRABP-specific hybridization signal has extended to nearly the whole outer neural cell layer, as shown in Figure 9. In embryos of this stage, HNK-1 expression was observed all along the neural tissue.

In the craniofacial mesenchyme, CRABP expression is still detectable, but reduced as compared to stage 22 embryos. Expression in the visceral arch area is similarly diminished, with the strongest hybridization signal appearing at the ventral side of the

arches. At this stage, neither the craniofacial mesenchyme nor the visceral arches showed any immunoreactivity with HNK-1. An elevated level of CRABP transcripts was still detected in the spinal cord, and in the dorsal root ganglia and cranial ganglia, which showed a continuously strong expression of the HNK-1 epitope. Figure 10 shows a transverse section through the spinal cord, with a characteristic distribution of CRABP transcripts on the dorsolateral sides and in two cell groups located ventrally, near the notochord.

Figure 3

Localisation of CRABP transcripts in a longitudinal section from a stage 12 (day 2) embryo. CRABP positive cells, indicated by arrows, appear in the CNS, the craniofacial mesenchyme, and the auditory pit (A). An elevated hybridization signal is also observed in the presumptive neural crest cells that are shown to be HNK-1 positive in Figure 4 (open arrow).

Figure 4

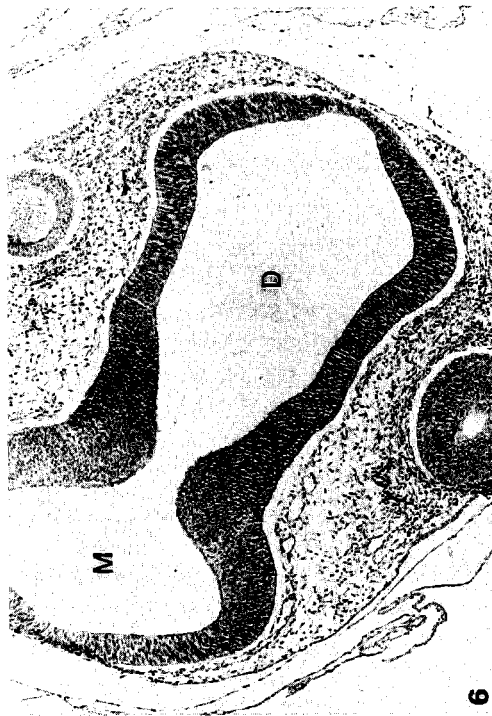
HNK-1 immunoreactivity in a section serial to the one shown in Figure 3. HNK-1 positive cells appear in the craniofacial mesenchyme, the auditory pit (A). A group of HNK-1 positive cells, presumably neural crest cells, is observed anterior to the auditory pit (open arrow).

Figure 5

Transverse section from the head region of a stage 22 (day 3) embryo, with CRABP-specific hybridization in the CNS and the craniofacial mesenchyme (arrows). D, diencephalon; M, mesencephalon.

Figure 6

HNK-1 immunoreactivity in a transverse section from a stage 22 (day 3) embryo, serial to the section shown in Figure 5. Positive cells are present in the CNS, and in the lateral parts of the head mesenchyme, mainly in the area surrounding the eyes. D, diencephalon; M, mesencephalon.



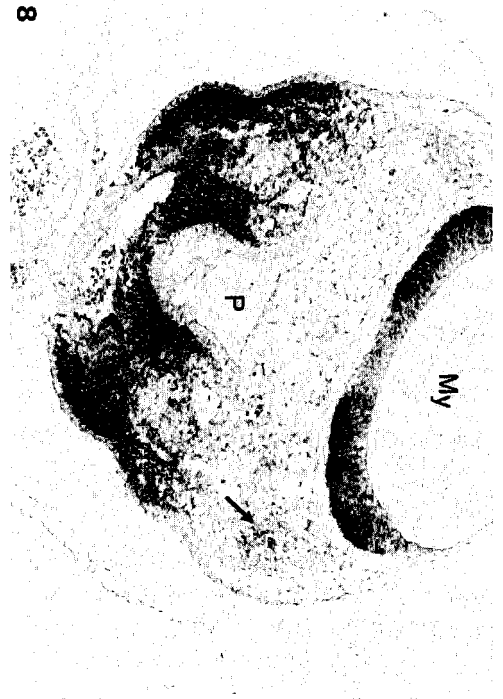
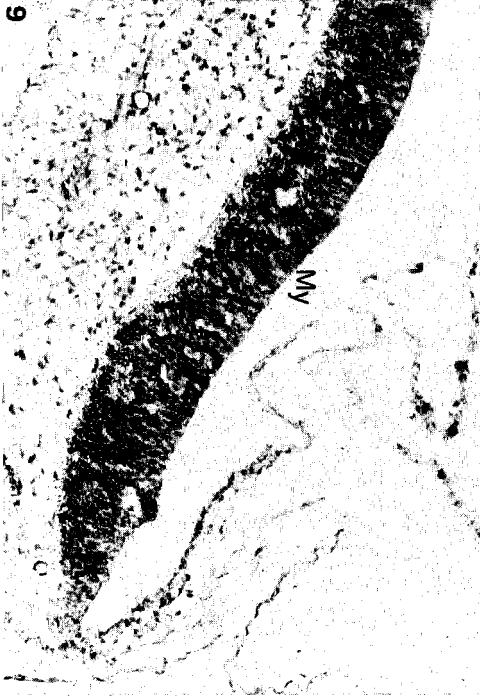


Figure 7

CRABP-specific hybridization in a longitudinal section from a stage 22 (day 3) embryo, showing CRABP expression in the visceral arch area, and in the auditory vesicle (A). I, II, III, first, second, and third visceral arch.

Figure 8

Transverse section from a stage 22 (day 3) embryo, demonstrating CRABP expression in the mantle layer of the myelencephalon (My), in the first visceral arch, and in the fifth cranial ganglion (arrow). P, pharynx.

Figure 9

CRABP-specific hybridization in a transversal section from a stage 24 (day 4) embryo, with positive cells in the mantle layer of the myelencephalon (My).

Figure 10

Transversal section from a stage 24 (day 4) embryo, showing CRABP expression in the neural tube and in a dorsal root ganglion (open arrow). The CRABP positive cells located on the ventral side of the neural tube are indicated by solid arrows. N = notochord.

Discussion

We have isolated a cDNA clone encoding part of chicken CRABP and used it for *in situ* hybridization studies of CRABP expression during chicken embryogenesis. After determination of the nucleotide sequence of our partial cDNA clone ChCRABP C4.5, we compared the deduced amino acid sequence with mouse CRABP (Vaessen *et al.*, 1989a). Only 6 out of 100 amino acids were different, with most substitutions concerning amino acids with similar physicochemical properties. The high degree of conservation observed for CRABP in cow (Sundelin *et al.*, 1985), mouse, and chicken suggests that the biological function of the protein does not allow important structural variation. The protein sequence of ChCRABP C4.5 is in agreement with the partial sequence of chicken CRABP type I as reported by Kitamoto *et al.* (1988). These authors state that two types of CRABP containing 6 amino acid replacements in the NH₂ terminal region are present in the chick embryo: a major one, type I, and a minor one, type II. It would be of interest to know whether CRABP I and II are encoded by different loci, or are the result of e.g. differential mRNA splicing. However, Southern blotting experiments performed on mouse and chicken DNA as well as on DNA isolated from mouse-hamster and human-hamster somatic cell hybrids did not, so far, give any indication for the existence of more than one CRABP gene (Vaessen *et al.*, 1989a, b).

Our *in situ* data demonstrate that CRABP expression in the CNS is strictly limited to a subpopulation of neural cells in day 2, day 3 and day 4 embryos. CRABP expression begins in a few single cells, gradually expanding to the whole outer cell layer of mesencephalon, rhombencephalon and spinal cord. In agreement with the results reported earlier for the mouse embryo, we also detected a high level of CRABP

transcripts in cells of the craniofacial mesenchyme. In addition, we here report that CRABP is highly expressed in the visceral arches. As a matter of fact, CRABP expression in the visceral arch area also occurs in the mouse embryo (Vaessen, unpublished results). Thus, CRABP expression patterns in chicken and in mouse embryos show a strong similarity.

The major CRABP expression sites reported here exhibit a striking correspondence to tissues commonly affected by exposure of mammalian embryos to RA. Clinical reports include malformations of the CNS - mainly hydrocephalus; of structures derived from the craniofacial and mandibular arch mesenchyme - microtia/anotia, micrognathia and cleft palate; congenital heart defects - predominantly conotruncal or branchial-arch mesenchymal-tissue defects, occasionally combined with thymic defects (Lammer *et al.*, 1985). Although the adverse effects of RA on the chicken embryo are less well documented, the target tissues seem to be the same (Jelinek and Kistler, 1981). Several reports implicate Vitamin-A in the inhibition of cranial neural crest cell development in the chick embryo (Hassell *et al.*, 1977; Keith, 1977). In addition, recent results obtained in our own group demonstrate that RA treatment of chicken embryos may give rise to congenital heart defects and craniofacial malformations as well as to limb deformations (M. Broekhuizen, pers. comm.). Apart from leading to digit duplications, local application of RA to the chick wing bud may also result in upper beak defects (Tickle *et al.*, 1982; Tamarin *et al.*, 1984; Wedden and Tickle, 1986). Interestingly, after treatment with RA we also obtained an embryo with a cleft lower beak (Vaessen, unpublished results), suggesting that in the chicken embryo the first arch - the lower beak primordium - is susceptible to the teratogenic effects of RA, just as in the mammalian embryo. The fact that Wedden and Tickle did not observe effects of RA on the lower beak must probably be ascribed to their using local application of RA in the chick wing bud as a means to generate malformations. In conclusion, the resemblance of CRABP expression patterns between chicken and mouse embryos, and the fact that in both species the CRABP-positive tissues appear to be susceptible to the adverse effects of RA, leads to the assumption that similar RA-sensitive morphogenetic processes take place in both species, and that CRABP expression has an important function in these processes.

The tissues that are frequently affected by RA-induced malformations share a common embryonic origin in that they have all received contributions from the cephalic neural crest. It has been suggested that excess RA has an adverse influence on cephalic neural crest cells, possibly by interfering with normal neural crest cell migration (Thorogood *et al.*, 1982; Pratt *et al.*, 1987). This theory is supported by the observation that RA interferes with the cell - substratum adhesion of neural crest cells *in vitro* (Smith-Thomas *et al.*, 1987). If endogenous RA concentrations also determine the migratory behaviour of neural crest cells *in vivo*, differential expression of CRABP could

play a role in the regulation of this process. Maden *et al.* (1989) described the occurrence of single CRABP-positive cells in a line from the dorsal neural tube to the lateral edge of the dorsal aorta and suggest that these cells may be neural crest cells in the progress of migration. The fact that these CRABP-expressing cells have escaped our attention can be explained by immunocytochemistry being better suited for detection of single positive cells than *in situ* hybridization, due to the higher background levels obtained with the latter technique. However, we observed two additional CRABP expression sites that may be related to neural crest cell migration: the craniofacial mesenchyme and the visceral arch area.

For a more detailed investigation of a possible relationship between CRABP expression sites and neural crest cell migration, we employed the monoclonal antibody HNK-1 (Abo and Balch, 1981), which recognizes an epitope present on several cell adhesion molecules. Canning and Stern (1988) showed that HNK-1 identifies tissues involved in mesoderm formation in the chick embryo. Prior to mesoderm induction, HNK-1 binds to the inducing tissue (hypoblast) and reveals a mosaic pattern in the responding tissue (epiblast). After primitive streak formation, the epiblast displays an anteroposterior gradient of HNK-1 expression. At the end of gastrulation, the primitive streak region loses its HNK-1 reactivity. HNK-1 expression is next seen in cells of the forming notochord and in cranial neural crest cells (Canning and Stern, 1988; Stern and Canning, 1990). In later stages of chicken embryogenesis, HNK-1 recognizes most premigratory neural crest cells and neuronal neural crest cell derivatives (Vincent *et al.*, 1983; Vincent and Thiery, 1984; Tucker *et al.*, 1984). However, the absence of HNK-1 immunoreactivity does not rule out the possibility that migratory neural crest cells are present since disappearance of the HNK-1 epitope from cranial neural crest cells at certain stages of migration has been reported (Vincent and Thiery, 1984; Bronner-Fraser, 1987).

In view of the existence of multiple HNK-1 antigens during development and adult life, it might seem surprising that migrating neural crest cells in the avian embryo can be visualized specifically with HNK-1 or related antibodies. Although reactivity is not restricted to neural crest cells, use of HNK-1 as a marker for neural crest cells is possible because the staining of other antigenic lineages does not overlap topographically or temporally with the distribution of crest cells. For one thing, neural crest cells do not become HNK-1 positive until they leave the neuroepithelium and start to migrate. Additional proof for the presence of the HNK-1 epitope on migrating neural crest cells is the fact that injection of HNK-1 antibodies lateral to the mesencephalic neural tube perturbs cranial neural crest cell migration (Bronner-Fraser, 1987).

In our investigation of a possible relationship between CRABP expression and neural crest cells we found that the cranial and dorsal root ganglia are positive for both

CRABP and HNK-1. In the CNS, a partial coincidence of CRABP and HNK-1 expression is observed, with a remarkable colocalization at stage 22. In stage 24 embryos CRABP continues to be differentially expressed, while HNK-1 reactivity occurs all along the neural tissue. However, the observed expression of the HNK-1 epitope in the CNS is probably unrelated to neural crest cell migration.

The cells of the craniofacial mesenchyme are initially (stage 12) positive for HNK-1 and also express CRABP. Later during development (stage 22) they start to differentiate into cartilage, muscle and bone, and lose the HNK-1 epitope. In contrast, CRABP expression in the craniofacial mesenchyme continues until stage 24. The visceral arches are filled with cells of neural crest origin (Le Lièvre and Le Douarin, 1975) which contribute to the development of the heart and arch arteries (Bockman *et al.*, 1987; Philips *et al.*, 1987). While showing a high level of CRABP expression, these neural crest derived cells do not express the HNK-1 epitope anymore. After administration of RA to chicken embryos Jelinek and Kistler (1981) showed that treatment on day 3 frequently gave rise to heart defects. Administration on day 4 resulted in a high incidence of heart defects and craniofacial malformations, as well as limb deformations. A tentative suggestion is evoked that the period of sensitivity to RA treatment of a particular morphogenetic system coincides with CRABP expression and loss of HNK-1 reactivity.

The role of CRABP in RA-mediated morphogenetic processes is still poorly understood. It has been suggested that CRABP has a function as a transport protein, mediating transfer of RA to the nucleus, where RA is thought to exert its biological activity (Takase *et al.*, 1986; Shubeita *et al.*, 1987). On the other hand, the human myelocytic leukemia cell line HL60, which is deficient in CRABP (Breitman *et al.*, 1982; Douer and Koeffler, 1982), is still able to differentiate in response to RA, indicating that binding to CRABP is not obligatory for transport of RA to its nuclear receptor sites.

A different model, proposed by Hirschel-Scholz *et al.*, suggests that CRABP has a buffer function, protecting the cell from the deleterious effects of unbound RA (Hirschel-Scholz *et al.*, 1989). Maden *et al.* reason along the same lines, proposing a function for CRABP in the sequestering of RA in the cytoplasm (Maden and Summerbell, 1989). The existence of reciprocating concentration gradients for RA and CRABP in the developing chick limb bud (Thaller and Eichele, 1987; Maden *et al.*, 1988) is consistent with these models. While the concentration of RA itself is highest in the posterior part, CRABP is present in a higher concentration in the anterior part. Thus, CRABP could be effective in reducing the concentration of free RA in the anterior part.

Recently, a nuclear receptor for RA (RAR α) was identified, which was shown to be related to the steroid/thyroid hormone receptor family (Petkovich *et al.*, 1987; Giguère *et al.*, 1987). In addition, three more RA receptors were identified, designated

RAR β , RAR γ , and RAR δ , which were related to RAR α but were obviously encoded by different genes (Brand *et al.*, 1988; Krust *et al.*, 1989; Ragsdale *et al.*, 1989).

It is assumed that the different RA receptors act as transcription factors, mediating expression of specific sets of genes. This suggests that RA-induced malformations occur as the result of aberrant gene expression. Evidently, genes known from *in vitro* experiments to be susceptible to RA induction, such as the gene encoding Growth Hormone (Bedo *et al.*, 1989), and the homeobox-containing genes (Colberg-Poley *et al.*, 1985a, b; Breier *et al.*, 1986; Deschamps *et al.*, 1987; Mavilio *et al.*, 1988), are candidate target genes involved in RA-induced malformations.

We propose that a high level of CRABP expression in certain tissues reflects a particular sensitivity to RA. CRABP would be instrumental in protecting cells from the developmentally important action of RA by prohibiting aberrant activation of RA responsive gene sequences during critical stages. This would explain why fetal exposure to excess RA predominantly affects those tissues that exhibit a high level of CRABP expression during certain stages of embryogenesis.

Acknowledgements

The authors thank R. Beekhuizen for technical assistance. This work was supported by the Netherlands Cancer Society (Koningin Wilhelmina Fonds).

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Chapter V

EMBRYONAL CARCINOMA-DERIVED CELL LINES EXPRESSING DIFFERENT LEVELS OF CELLULAR RETINOIC ACID-BINDING PROTEIN EXHIBIT DIFFERENTIAL RESPONSIVENESS OF THE RETINOIC ACID RECEPTOR β GENE TO INDUCTION BY RETINOIC ACID.

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submitted for publication

Abstract

The cell lines MES-1 and END-2 are both differentiated derivatives from the embryonal carcinoma cell line P19, but MES-1 cells contain a high level of cellular retinoic acid-binding protein (CRABP) mRNA, whereas END-2 cells are CRABP-deficient. In view of a possible role of CRABP in retinoic acid (RA)-mediated signal transduction, we have studied the RA-induced up-regulation of the retinoic acid receptor β gene in the cell lines END-2 and MES-1. Comparison of the results obtained with both cell lines suggests that a high level of CRABP impedes the cellular response to RA. In addition, we show that exposure of MES-1 cells to RA results in a decrease in the level of CRABP mRNA.

Introduction

The vitamin A derivative retinoic acid (RA) has long been known to have profound effects on cellular differentiation and proliferation processes. More recent evidence

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indicates that RA functions as a morphogen in embryonic pattern formation. When administered to the developing chick limb bud, RA has the ability to stimulate the production of additional digits (Tickle *et al.*, 1982; Summerbell, 1982). Moreover, endogenous RA is present in the developing chick limb bud, where it forms a concentration gradient across the anteroposterior axis (Thaller and Eichele, 1987). RA is thought to exert its biological activity through the activation of nuclear RA receptors (RARs), which belong to the superfamily of steroid/thyroid hormone receptors, and may act as inducible transcriptional enhancer activators (for review, see Evans, 1988).

Several lines of evidence implicate the cellular RA binding protein (CRABP) in RA action. Structure-activity studies show that the affinity with which specific retinoid analogues bind to CRABP correlates well with their biological activity (Jetten and Jetten, 1979; Lotan, 1980; Sani *et al.*, 1984; Keeble and Maden, 1986). In addition, CRABP is preferentially expressed in embryonic tissues that are susceptible to RA induced malformations (Maden *et al.*, 1989; Perez-Castro *et al.*, 1989; Vaessen *et al.*, 1989, 1990). In the limb bud, CRABP exhibits a graded distribution (Maden *et al.*, 1988; Dollé *et al.*, 1989), indicating that differential expression of CRABP could be involved in the generation of the putative morphogenetic field. However, the exact role of CRABP in RA action is not quite understood. CRABP was originally thought to have a transport function, facilitating transport of RA to the nuclear receptor sites (Jetten and Jetten, 1979; Schindler *et al.*, 1981; Takase *et al.*, 1986). A different hypothesis was based on the observation that the CRABP gradient in the developing limb bud is of opposite polarity to that of RA itself (Maden *et al.*, 1988; Dollé *et al.*, 1989). Consequently, Maden *et al.* suggested that CRABP is involved in lowering the effective concentration of unbound RA, thus steepening the morphogenetic RA gradient (Maden *et al.*, 1989).

Treatment of cultured cells with RA has been shown to result in induction of the RAR β gene (De Thé *et al.*, 1989; Song and Siu, 1989; Hu and Gudas, 1990). In order to gain more insight in the physiological function of CRABP, we have compared RA-induced transcriptional up-regulation of RAR β in murine embryonal carcinoma (EC) derived cell lines containing different levels of CRABP. Our results suggest that the cellular response to RA is impeded by a high level of CRABP. Rather unexpectedly, we found that in an EC cell line containing a high level of CRABP transcripts, prolonged exposure to RA results in a decrease in the amount of CRABP mRNA.

Materials and Methods

Cell lines and culture techniques

MES-1 and END-2 cells (obtained from Dr. Christine Mummery, Hubrecht Laboratory, Utrecht) were cultured in a 1:1 mixture of Dulbecco's Minimal Essential Medium and Ham's F10 medium, supplemented with 7% fetal calf serum, in 5% CO₂.

For comparison of RA response, identical culture flasks were seeded from the same batch of cells and grown to semi-confluence. In each flask, 50 µl of All-*trans*-RA (Sigma) stocks, prepared in dimethylsulfoxide, was added to 50 ml of culture medium. Control cells were treated with 50 µl dimethylsulfoxide.

RNA isolation and blot hybridization

Total RNA was isolated from cell lines using the LiCl/Urea method (Auffray and Rougeon, 1980). For RNA blot analysis, RNA was electrophoresed on 1% agarose gels in the presence of formaldehyde (Maniatis *et al.*, 1982). Prior to electrophoresis, ethidium bromide was added to the RNA samples in order to allow visualisation of the ribosomal RNAs in the gel. In this way, it was ascertained that the amounts of RNA in the different lanes were approximately the same.

The RAR β probe was a 660 bp. *Eco*RI fragment of the human RAR β cDNA clone pCOD20 (De Thé *et al.*, 1987). For detection of CRABP mRNAs, we used a 170 bp. *Taq*I-*Taq*I fragment of mouse CRABP cDNA clone MoT-CA11 (Vaessen *et al.*, 1989).

The DNA probes were labelled with ³²P using random priming (Feinberg and Vogelstein, 1983), and hybridized to the RNA blots at 42°C in a buffer containing 9% dextran sulphate and 50% formamide. The filters were washed at 65°C, twice in 3 xSSC, 0.1% NaDodSO₄ and twice in 1xSSC, 0.1% NaDodSO₄.

Results and Discussion

CRABP expression in P19 EC derived cell lines

P19 is a murine EC cell line capable of *in vitro* differentiation into many cell types. Permanent clonal cell lines have been established representing the ectodermal (EPI-7), endodermal (END-2) and mesodermal (MES-1) line of differentiation (Mummery *et al.*, 1985, 1986). Previously, we have demonstrated that the CRABP gene is abundantly

expressed in MES-1 cells as compared to undifferentiated P19 EC cells (Vaessen *et al.*, 1989). RNA blot analysis of the other P19 derivatives revealed that a low level of CRABP mRNA is expressed in EPI-7 cells, whereas no CRABP transcripts could be detected in END-2 cells (data not shown).

Because of the extreme difference in CRABP expression between MES-1 and END-2 cells, we chose to compare these cell lines with respect to transcriptional up-regulation of the RAR β gene following RA treatment.

Dose-response of RA-induced RAR β expression

To investigate whether the presence or absence of CRABP has any effect on the RA concentration needed to obtain transcriptional activation of RAR β , we have treated MES-1 and END-2 cells with varying concentrations of RA, taking care to standardize culture conditions for all samples. Semi-confluent cells were cultured in medium to which the appropriate amount of RA was added, and harvested after 24 hours. Total cellular RNA was isolated, and analyzed for the presence of RAR β mRNA by blot hybridization.

As can be seen in Figure 1A, RAR β transcripts are present in END-2 cells grown in normal medium, without the addition of extra RA. It should be noted, however, that the medium used for culturing the cells may have contained trace amounts of RA, as it was not charcoal-stripped. Treatment of END-2 cells with increasing amounts of RA results in elevated levels of RAR β expression, showing significant stimulation by RA concentrations as low as 10^{-9} - 10^{-8} M. This RA-associated increase in RAR β mRNA is in agreement with the results described by others. De Thé *et al.* (1989) reported RAR β induction in human hepatocytes at a RA concentration of 10^{-9} M, whereas Hu and Gudas (1990) obtained similar results using F9 cells.

In MES-1 cells, RA induction of RAR β follows a different pattern, as is illustrated by Figure 1B. When the cells are grown in medium containing RA concentrations of up to 10^{-8} M, RAR β is expressed at a very low level. At 10^{-7} M RA some induction occurs, but a really dramatic increase of RAR β expression is observed after treatment with 10^{-6} M RA. At this concentration, RAR β expression is elevated to a level comparable to that observed in END-2 cells after RA exposure. This result is suggestive of a function for CRABP in absorption of RA, preventing RA from reaching the nucleus. At 10^{-6} M RA, the amount of cytoplasmic binding protein would not be sufficient to absorb all the supplied RA, which would result in diffusion of RA to the nucleus and subsequent induction of the RAR β gene.

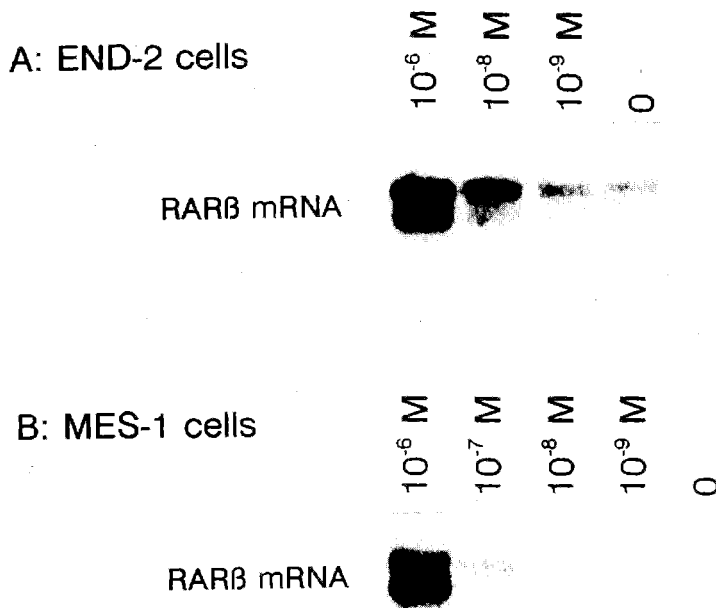


Figure 1

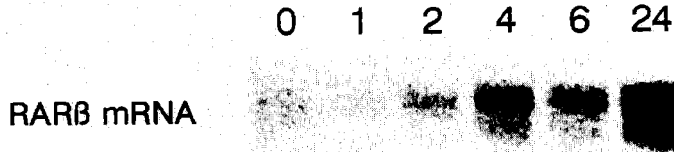
Dose-response of RA-induced RARβ gene expression in (A) END-2 and (B) MES-1 cells.

Time-response of RA-induced RARβ expression

To investigate whether the level of CRABP influences the interval between application of RA and induction of RARβ expression, we performed a time-course experiment. END-2 and MES-1 cells were cultured in the presence of 10⁻⁶ M RA for varying lengths of time, and harvested immediately afterwards. Up-regulation of RARβ gene expression in END-2 cells has a latency of 2 hours and is complete after 6-24 hours of RA treatment (Figure 2A). In MES-1 cells induction of RARβ mRNA is first observed after 4 hours and reaches its maximum after 4-24 hours (Figure 2B).

The observed latencies of RARβ induction agree with the results reported by the Thé *et al.* (1989), who observed RARβ induction after 4 hours RA treatment of human hepatocytes. Comparison of the data we obtained using the two cell lines shows that the response to RA in MES-1 occurs with the same speed or even slower than in END-2. This result again opposes the idea that CRABP mediates transport of RA to the nucleus.

A: END-2 cells



B: MES-1 cells

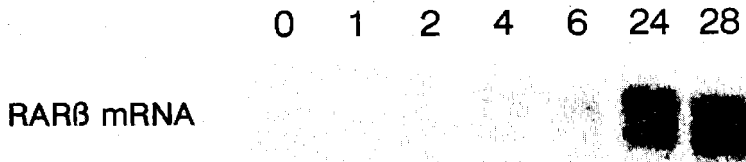


Figure 2

Time-response of 10^{-6} M RA-induced RAR β gene expression induced in (A) END-2 and (B) MES-1 cells (time in hours).

Alterations in CRABP expression

To illustrate the differences in CRABP expression in the various RNA samples that were analyzed for RA-associated RAR β induction, the blots shown in Figures 1 and 2 were hybridized to a CRABP probe. No CRABP transcripts were detected in the RA-treated END-2 cells, but in MES-1 cells RA treatment resulted in alteration of the level of CRABP mRNA. As can be seen in Figure 3A, the level of CRABP transcripts in RNA samples isolated from MES-1 cells treated with various concentrations of RA was substantially reduced after growth in the presence of 10^{-7} M RA. At 10^{-6} M RA, the decrease in CRABP mRNA level is even more pronounced (see Figure 3A). The filters containing RNA samples from the time-course experiment were also hybridized with a CRABP probe. As illustrated by Figure 3B, the level of CRABP expression in RA-treated MES-1 cells remains approximately the same for the first 24 hours, and decreases in the period from 24 to 28 hours.

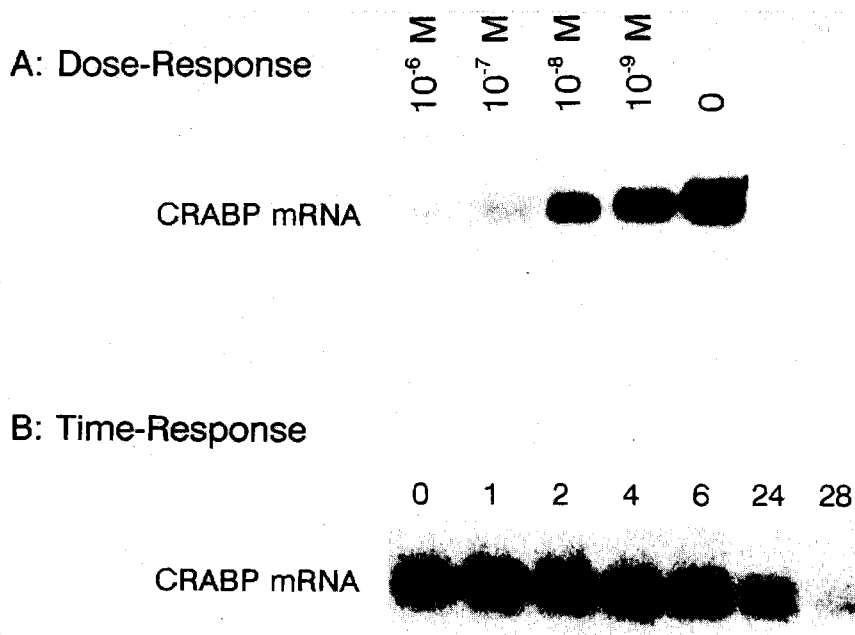


Figure 3
Alterations in CRABP expression after treatment of MES-1 cells with RA. A: dose-response after 24 hours of RA exposure, and B: time-response after exposure to 10^{-6} M RA (time in hours).

In spite of some experimental variation in time of onset, probably due to slight differences in cell density and RA concentration, it is clear that RA treatment of MES-1 cells results in a reduced level of CRABP transcripts, which becomes apparent only after prolonged incubation. If CRABP indeed functions as a storage protein for RA, this result is surprising, since addition of RA might have been expected to give rise to accumulation of CRABP to sequester the ligand. Also, others have reported that CRABP expression is elevated in P19 or F9 EC cells after induction with RA (Eriksson *et al.*, 1986; Wei *et al.*, 1987; Stoner and Gudas, 1989). The discrepancy must be due to the fact that the steady-state level of CRABP mRNA is much lower in the undifferentiated EC cells than in MES-1, where CRABP appears to be already induced. The observed decrease in the level of CRABP mRNA after RA treatment of MES-1 cells suggests that in these cells feed-back mechanisms are activated once RA reaches the nucleus. Considering the fact that the MES-1 cell line is derived from P19 EC, and presumably retains some

embryonal characteristics, these regulation mechanisms may also be operative in the embryo itself.

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Chapter VI

ANALYSIS OF THE PROMOTER REGION OF THE CELLULAR RETINOIC ACID-BINDING PROTEIN GENE: TRANSCRIPTIONAL REGULATION REQUIRES THE PRESENCE OF ENHANCER ELEMENTS.

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Abstract

The gene encoding cellular retinoic acid-binding protein (CRABP) exhibits a spatio-temporally specified expression pattern during embryogenesis. In an attempt to identify *cis*-acting elements that control CRABP gene expression, we have isolated mouse genomic DNA containing the CRABP gene, and determined the nucleotide sequence of a region upstream of the transcriptional initiation site. Via reporter gene analysis in CRABP-expressing cells, we demonstrated that this region contains promoter activity. However, this promoter by itself is not sufficient to drive high levels of expression, but apparently requires the presence of additional as yet unidentified enhancer elements.

Introduction

Pattern formation in embryonic development is assumed to be governed by concentration gradients of specific signal molecules, generally referred to as morphogens. Retinoic acid (RA), a physiological metabolite of vitamin A, has been shown to affect various embryonic processes, including axis formation (Mitrani and Shimoni, 1989; Sive *et al.*, 1990), development of the central nervous system (Durston *et al.*, 1989), and pattern formation in the limb (reviewed by Eichele, 1989). The actual demonstration of

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differential levels of endogenous RA across the anteroposterior axis of the limb bud (Thaller and Eichele, 1987) suggests that RA may be an important natural morphogen.

RA is thought to exert its biological effects through transcriptional activation of developmental control genes, brought about by interaction of RA with specific nuclear receptors (Evans, 1988). Apart from these nuclear RA receptors, the RA-induced signal transduction pathway also seems to involve the cellular retinoic acid-binding protein (CRABP), a small cytosolic protein that has a highly specific binding affinity for RA (Ong and Chytil, 1975; Jetten and Jetten, 1979; Lotan, 1980; Sani *et al.*, 1984; Keeble and Maden, 1989). The CRABP gene exhibits a spatio-temporally specified expression pattern during embryogenesis which correlates well with the apparent sensitivity to RA of the tissues concerned (Maden *et al.*, 1989; Vaessen *et al.*, 1990). Hence, it has been suggested that differential expression of CRABP could be involved in the generation of the putative morphogenetic fields. Such an important function would obviously necessitate a strict regulation of the CRABP gene, presumably taking place at the transcriptional level. In this study, we describe a preliminary characterization of the CRABP promoter region. Our results show that the minimal promoter sequences are not sufficient to drive high level transcription, suggesting that the spatio-temporally specified expression of the CRABP gene is mediated via additional regulatory elements.

Materials and Methods

DNA blot analysis

Following cleavage with restriction enzymes, DNA fragments were separated by electrophoresis on agarose gels, and transferred onto nitrocellulose filters by Southern blot techniques (Southern, 1975). DNA probes, labelled with ³²P using random priming as described by Feinberg and Vogelstein (1983) were hybridized at high stringency to the DNA blots. The filters were washed at 65°C, twice in 3xSSC, 0.1% SDS, twice in 1xSSC, 0.1% SDS, and once in 0.3xSSC, 0.1% SDS..

Isolation of genomic clones containing mouse CRABP DNA

A mouse genomic library in bacteriophage vector EMBL3 (generated by A. van Agthoven) was screened with a 170 bp. *TaqI-TaqI* fragment of the mouse CRABP cDNA clone MoT-CA11 (Vaessen *et al.*, 1989). This resulted in the isolation of several overlapping positive clones. Restriction mapping was carried out with single and double restriction enzyme digestions, and hybridization with fragments of the CRABP cDNA was

investigated. On the basis of these results, several fragments were subcloned into plasmid vector pTZ18R (Pharmacia).

Nucleotide sequence analysis

For determination of the nucleotide sequence, DNA fragments were cloned in the plasmid vector pTZ18R or pTZ19R (Pharmacia). After infection of the resulting recombinant clones with m13 helper bacteriophages, single stranded DNA was isolated. The DNA sequence was determined via the dideoxy chain termination method (Sanger *et al.*, 1977), using Sequenase (United States Biochemical, Cleveland, Ohio) according to instructions by the manufacturer.

5'-End amplification of CRABP cDNA

Total RNA was isolated from MES-1 cells using the LiCl Urea method described by Auffray and Rougeon (1980). 5 μ g of MES-1 RNA was reverse transcribed using MuMLV-reverse transcriptase (BRL) according to instructions by the manufacturer. cDNA synthesis was primed with a 22-mer oligonucleotide GCGCAGCAGCGAGAATTCCGACG, which corresponds to nucleotides 127-149 of the CRABP cDNA sequence of MoT-CA11 (nucleotides 857-879 of the Sac880 sequence, see Figure 2), but containing a single mismatch, thus generating an *EcoRI* site.

Amplification of the resulting cDNA was performed according to the RACE-protocol (Frohman *et al.*, 1988). Following two cycles of Centricon centrifugation (Amicon) to remove excess primers, the cDNA was poly(dA)-tailed using TdT (Boehringer). The poly(dA)-tailed cDNAs were amplified by PCR using *TaqI* polymerase (Cetus Corp., Emeryville, CA) as described by Hermans *et al.* (1987). The CRABP-specific 22-mer GCGCAGCAGCGAGAATTCCGACG (see above) and a mixture of GTCGCGAATTCGTCGACGCGTTTTTTTTTTTTTTTTT (PL-(dT)15) and GTCGCGAATTCGTCGACGCG (adaptor) were used as amplification primers. We performed 28 cycles of PCR in a DNA Thermal Cycler (Perkin-Elmer-Cetus). Denaturation was achieved by heating the samples for 7 min. at 94°C, followed by 5 min. annealing time at 55°C. Subsequently, 5 cycles of extension (4 min. 72°C), denaturation (2 min. 94°C), and annealing (2 min. 55°C) were carried out, followed by an additional 23 cycles in which the annealing temperature was raised to 60°C. 10% of the reaction mixture was submitted to another 24 cycles of PCR, using the CRABP-specific 22-mer and the adaptor as amplification primers. PCR conditions were essentially the same as described above, but the annealing temperature was 60°C, and extension was carried on for 3 min.

The PCR-products were analyzed by Southern blot analysis, using the CRABP cDNA as a probe in hybridization. cDNA fragments of the size corresponding to the CRABP-positive band were purified by preparative agarose gel electrophoresis. This material was used for another round of PCR, again followed by preparative agarose gel electrophoresis. Finally, the cDNA was digested with *EcoRI*, and cloned in pTZ18R.

Construction of pSX1000

HE3200 plasmid DNA was linearized with *EcoRI*, and used as a template for PCR. The 5' amplification primer used for this reaction was a 25-mer ACCCGCGTCTTGCGGTCAGATCTCG, which is complementary to nucleotides 716-736 of the Sac880 sequence, plus an additional 5 nucleotides constituting an *XbaI* site. The 3' amplification primer was a 38-mer CGAACCGTGACCGGCAGCAAGGGCCCTAGGCAGCTGGC, which comprises 20 nucleotides of the pTZ18R-sequence and a stretch of non-homologous nucleotides generating recognition sites for *SalI*, *BamHI*, and *SmaI*. The PCR was carried out essentially as described for the first round of 5'-end amplification. After removal of excess primers by Centricon centrifugation, the amplified DNA was digested with *SalI* and *XbaI*, and cloned in pTZ18R.

Chloramphenicol acetyltransferase assay

MES-1 cells (obtained from Dr. Christine Mummery, Hubrecht Laboratory, Utrecht) were cultured in a 1:1 mixture of Dulbecco's Minimal Essential Medium and Ham's F10 medium, supplemented with 7% fetal calf serum, in 5% CO₂. The cells were seeded at a density of 1 x 10⁶ per 6 cm. dish 16 hours prior to transfection. Using the calcium phosphate precipitation procedure (Graham and van der Eb, 1973) each dish was transfected with 5 µg of CAT-containing plasmid, and 1 µg of RSV-lacZ. Cells were harvested 40-48 hours after transfection. Subsequently, crude cell extracts were prepared by several freeze-thaw cycles. CAT activity and β-galactosidase activity were determined as described by Gorman (1985).

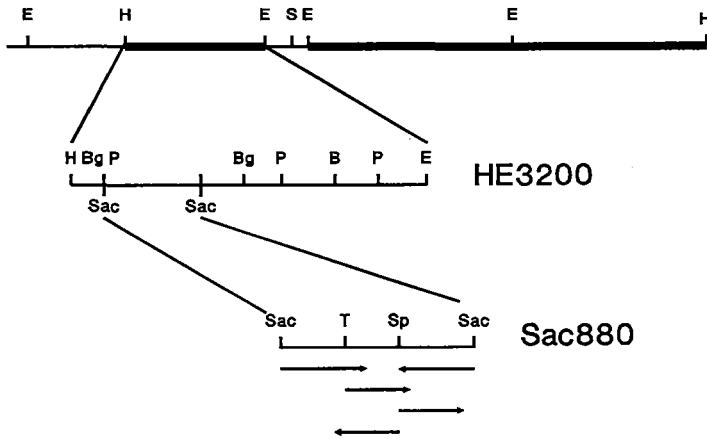


Figure 1

Physical maps of the mouse CRABP genomic DNA and of the subclones HE3200 and Sac880. The direction of transcription is from left to right. Bold lines mark hybridization with the CRABP cDNA. The sequencing strategy is indicated by arrows, shown beneath the Sac880 map. Restriction enzymes are abbreviated as follows: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; S, Sall; Sac, SacI; Sp, SpeI; P, PstI; T, TaqI.

Results

Isolation and partial characterization of mouse genomic CRABP clones

Previously, we have described the molecular cloning of a mouse CRABP cDNA, which was isolated from a mouse testis cDNA library (Vaessen *et al.*, 1989). To further characterize the mouse CRABP gene, and to study the regulation of CRABP gene expression, we have isolated mouse CRABP genomic DNA. A mouse genomic library, constructed in the bacteriophage vector EMBL3, was screened with a fragment of the mouse CRABP cDNA clone MoT-CA11. This resulted in the isolation of several overlapping clones. After digestion with various restriction enzymes, and Southern blot hybridization with ^{32}P -labelled fragments of the CRABP cDNA, we were able to construct a simple physical map of the mouse CRABP locus (see Figure 1). Based on these results, we subcloned a 3200 bp. *HindIII-EcoRI* fragment into plasmid vector pTZ18R. The resulting subclone HE3200, which presumably contains the 5' part of the CRABP gene, was mapped in more detail (Figure 1).

1	GAGCTCAAACCCGAATTTTACAAAATAAGCTGCCGATTTCCCTGCATTTTGCTGTTC	Sac880
61	CGTCCCTTAGGCAACAACAAGGGACGGTGTGGTCTGGGCTCCCTTCTAACTTAAGCAG	Sac880
121	AAAACCTGCATGAGAGGCTTGGTTTCCAGCACCCGTTTGAACCTGCCTTGGTACCTCCGT	Sac880
181	GCCTCCATGCCCGGATAAGCAAGGGTAGGGGCTGGGCCTTCCCGCCACTCTAATTGGT	Sac880
241	CCTCCATATGCTAACTGAAAAGCCGAACAGAGCAAATTCGACCCGGCTAGTGGCAGCCAGA	Sac880
301	AAGTATGGTGGAAAGAAAGTGGCTTCCATGCCTATGGCCTTACTACTAACAGATATTTT	Sac880
361	CAAACATAGGCAAAAAGTAGAGAAAGATGTCGCGTTCCTGAATCCCCTACTCTAGCT	Sac880
421	AGCCTCATGTATCTCCTCCAGGCCGACCTAGCTTCATTAACCCTCTCTACCACCCAGT	Sac880
481	TTAAAACAGAATGTGGCGCGTGGTGGAGGAGCACCAGGGAGAGCAAGTTCCAAGTT	Sac880
541	GCATCACTAGTGGCTGAGAAGATGAGGAATGGTGGGGGCGCCTTAGCGCGGAGTGG	Sac880
601	GCTCACCCCTCGTGGGCCACCCCGCCCTCGTGACGCAGGGCAGGGCGGACTTGA	Sac880
661	<u>GGCGGGGG</u> CCCCAGAGGGGGGGGGCTACCCTAGCGACTCAAGGCGCTTGC GGCTGGG	Sac880
	↓ ↓	
721	CGCAGAACGCCAGTCTCTCTGCAACGCAGCGTGCAGCCGCTCCGCAGCAGAGTGGGTGC	Sac880
1	CAGTCTCTCTGCAACGCAGCGTGCAGCCGCTCCGCAGCAGAGTGGGTGC	MoTCA11
781	CTGCCCTGCCTTCGCGCCCGCTACAGCCAACACCCTGCCACCATGCCCAACTTCGC	Sac880
51	CTGCCCTGCCTTCGCGCCCGCTACAGCCAACACCCTGCCACCATGCCCAACTTCGC	MoTCA11
841	CGGTACCTGGAAGATGCGCAGCAGCGAGAATTCGACGAGCTC	Sac880
111	CGGTACCTGGAAGATGCGCAGCAGCGAGAATTCGACGAGCTCCTCAAGGCGTGGGTGTG	MoTCA11
	MetArgSerSerGluAsnPheAspGluLeuLeuLysAlaLeuGlyVal	

Figure 2

Sequence of the 880 bp. *SacI-SacI* fragment containing the 5' part of the CRABP cDNA. The corresponding nucleotide sequence of the mouse CRABP cDNA clone MoTCA11 is shown underneath, with the N-terminal part of the CRABP protein sequence. Putative *SpI*-recognition sites are underlined. the transcriptional initiation sites as deduced from the primer extension-amplification experiment are indicated with arrows (↓).

The 5' part of the mouse CRABP cDNA maps within a *SacI-SacI* fragment of approximately 880 bp. This *SacI-SacI* fragment was subcloned into pTZ18R, and the nucleotide sequence was determined As illustrated by Figure 2, the *SacI-SacI* fragment contains 882 bp. of genomic DNA. It starts 730 bp. upstream of the 5' end of the CRABP cDNA sequence as known from MoT-CA11, and is colinear with the first 152 bp. of MoT-CA11, indicating that it does not reach as far as the first intron-exon boundary. The putative promoter region lacks TATA and CAAT boxes and is rich in GC residues.

Determination of the transcriptional start site

In order to localize the transcriptional initiation site of the CRABP gene, we performed a primer extension experiment, followed by 5'-end amplification of the resulting cDNAs according to the RACE protocol of Frohman *et al.* (1988). Briefly, this approach entails reverse transcription, primed with a gene-specific oligonucleotide, followed by poly(dA) tailing of the newly produced cDNAs. The resulting poly(dA)-tailed cDNAs are amplified via the polymerase chain reaction, using a gene specific oligonucleotide and a mixture of the oligonucleotides PL(dT)₁₅ and adaptor as amplification primers. As a source of RNA, we used the cell line MES-1, which is derived from the embryonal carcinoma cell line P19 and contains a high level of CRABP transcripts (Mummery *et al.*, 1986; Vaessen *et al.*, 1989). This experiment resulted in generation of cDNA fragments of approximately 200 bp., which were cloned into plasmid vector pTZ18R. We determined the nucleotide sequences of three independent cDNA clones that were acquired in this way. All three clones are colinear with the sequence of the 5' genomic *SacI-SacI* CRABP clone. The two longer clones extend 6 nucleotides beyond the 5' end of the CRABP cDNA sequence as determined from clone MoT-CA11. The third clone reaches exactly to the first residue of the latter sequence.

Generation of CRABP promoter / CAT reporter gene constructs

To investigate whether the 880 bp. *SacI-SacI* 5' CRABP genomic fragment indeed contains promoter activity, and possibly sequences that regulate tissue-specific expression, we cloned this fragment at the 5' side of the coding sequences of the bacterial chloramphenicol acetyl transferase (CAT) gene. As a recipient for the putative promoter fragment we used a vector that was generated by cloning a *HindIII-ApaI* fragment from pSV2CAT (Gorman *et al.*, 1982) lacking the SV40 promoter into the *HindIII-ApaI* sites of the plasmid Bluescript (KS⁺) (Stratagene) (Tsonis *et al.*, 1988). Insertion of the 880 bp. *SacI-SacI* 5' CRABP genomic fragment in the *SacI* site of this CAT vector yielded a clone which we named pSaCAT. The correct 5'-3' orientation of the putative promoter fragment was established.

To see whether the putative CRABP promoter sequences present in pSaCAT can drive expression of the CAT gene, we transfected the plasmid into MES-1 cells, which are known to express high levels of CRABP mRNA (Vaessen *et al.*, 1989). However, no CAT activity could be detected in extracts from cells transfected with pSaCAT. pRSVcat was included in this experiment as a positive control to make sure transfection of the MES-1 cells was sufficiently efficient to give rise to detectable levels of CAT. The Rous sarcoma virus long terminal repeat is known to direct accumulation of high levels of

functional mRNA in various eukaryotic cell types (Gorman *et al.*, 1982). Indeed, extracts prepared from MES-1 cells transfected with pRSVcat showed efficient conversion of ¹⁴C-labelled chloramphenicol to the acetylated form. Apparently, the regulatory elements present in pSaCAT are not sufficient to drive CAT expression in MES-1 cells.

To check for promoter activity in the 5' CRABP genomic region, we decided to generate new reporter gene constructs. As a vector, we used the pCAT-Enhancer plasmid (Promega), which contains the SV40 enhancer element but no promoter to drive transcription of the CAT gene. Since this vector offers a limited choice of cloning sites, we used PCR techniques to generate a CRABP genomic fragment that could be inserted in front of the CAT gene. The plasmid HE3200, which contains approximately 1000 bp. upstream of the CRABP CAP-site, was used as a template for the PCR. Since the 5' part of this larger CRABP genomic fragment has not been sequenced, we synthesized a 5'-PCR primer which is complementary to the sequence of pTZ18R immediately upstream of the *Hind*III site into which the insert had been cloned. A stretch of non-homologous nucleotides was added to this oligomer to generate restriction sites for *Sal*I, *Bam*HI, and *Sma*I. As a 3' primer, we used an oligonucleotide complementary to the sequence around the CRABP CAP-site, and containing 5 additional nucleotides, constituting an *Xba*I site. Using these oligonucleotides as PCR primers and *Eco*RI-digested plasmid HE3200 as a template, we amplified a fragment of approximately 1000 bp. The resulting fragment was digested with *Sal*I and *Xba*I, and cloned into plasmid vector pTZ18R to yield pSX1000. This new clone was cleaved with several restriction enzymes to check whether the insert contains the appropriate recognition sites. The *Bgl*II, *Pst*I, *Sac*I, and *Sma*I sites that are located in the genomic DNA were detected at the correct positions. Also, the *Xba*I site that was created at the 3' end was recovered. However, it appears that the 5' sites for *Sal*I, *Bam*HI, and *Sma*I were lost during cloning procedures.

The 1000 bp. genomic fragment containing the putative CRABP promoter was excised from pSX1000 by cleavage with *Hind*III and *Xba*I, and cloned in the vector pCAT-Enhancer to generate clone pC1000-CAT-Enh. The fragment was also cloned in the vector pBL-CAT TK⁻. This vector, which lacks promoter and enhancer sequences, was created by excision of a *Bam*HI-*Bgl*II fragment, containing the TK promoter, from pBL-CAT2. The resulting clone was named pC1000-CAT.

To investigate whether the 1000 bp. CRABP genomic fragment can drive transcription of the CAT gene in the presence or absence of the SV40 enhancer element, we performed an experiment in which pC1000-CAT-Enh and pC1000-CAT were transfected into MES-1 cells. The cell extracts were assayed for CAT activity, which yielded the result shown in Figure 3. A certain amount of CAT activity is observed in control extracts prepared from cells that were transfected with the vector plasmids pCAT-Enhancer and pBL-CAT TK⁻. However, transfection of pC1000-CAT-Enh clearly

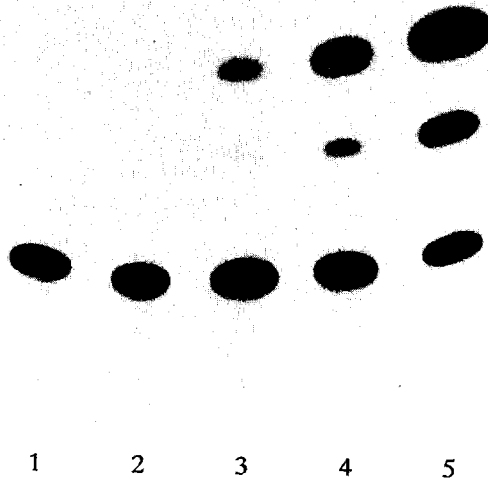


Figure 3

Chloramphenicol-transferase activity in MES-1 cells transfected with a mixture of 1 μ g RSV-lacZ and 5 μ g of the following plasmids: (1) pBL-CAT TK⁺; (2) pC1000-CAT; (3) pCAT-Enh; (4) pC1000-CAT-Enh; (5) pRSVcat.

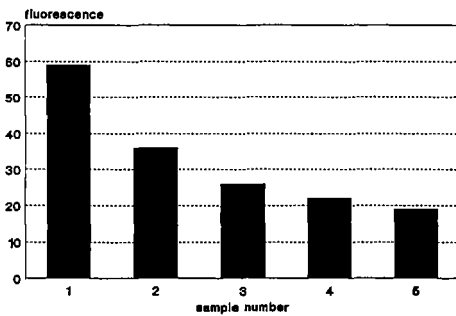


Figure 4

Relative transfection efficiency of the MES-1 cells used in the CAT assay as measured by β -galactosidase activity in different cell extracts. Cells were transfected with a mixture of 1 μ g RSV-lacZ and 5 μ g of the following plasmids: (1) pBL-CAT TK⁺; (2) pC1000-CAT; (3) pCAT-Enh; (4) pC1000-CAT-Enh; (5) pRSVcat.

gives rise to a higher CAT activity than that obtained with pCAT-Enhancer. In contrast to this, insertion of the CRABP genomic fragment in pBL-CAT TK⁻ does not lead to a stimulation of ¹⁴C-chloramphenicol conversion. In order to enable comparison of the transfection efficiencies of different DNA precipitates, plasmid pRSV-lacZ was cotransfected together with the CAT constructs. Determination of the β galactosidase activity in the cell extracts gave the results outlined in Figure 4. Although a strong variation in transfection efficiency is observed for different DNA samples, interpretation of the CAT assay is not altered. β galactosidase activity in extracts prepared from cells transfected with pC1000-CAT-Enh or pCAT-Enhancer is approximately the same. Although β galactosidase activity in the pBL-CAT TK⁻-sample is higher than in the pC1000-CAT-cell extract, the transfection efficiency obtained with the latter plasmid should have been sufficient to detect CAT activity. As a consequence, the observed lack of CAT activation after transfection of pC1000-CAT must be due to absence of the appropriate regulatory elements.

Discussion

We have isolated several genomic clones hybridizing to a mouse CRABP cDNA probe, and used these to compose a physical map of the mouse CRABP gene locus. Our map is in agreement with the hybridization pattern obtained by Stoner and Gudas (1989) using DNA isolated from the embryonal carcinoma cell line F9, and with the results obtained by Wei *et al.* (1990).

The nucleotide sequence of a *SacI-SacI* genomic fragment that hybridizes to the 5' part of the CRABP cDNA was determined. This fragment contains 150 bp. colinear with the CRABP cDNA sequence of the testis-derived cDNA clone MoT-CA11 (Vaessen *et al.*, 1989). Provided MoT-CA11 comprises the full length CRABP cDNA, the remaining 730 bp. should include the CRABP promoter sequences. The 5' end of the MoT-CA11 cDNA sequence coincides exactly with the transcription initiation site indicated by Shubeita *et al.* (1987) in the highly homologous bovine CRABP sequence. We verified the position of the mouse CRABP transcription initiation site by a PCR-amplified primer extension, using RNA isolated from MES-1 cells. This resulted in generation of cDNAs extending 5 bp. beyond the 5' end of the MoT-CA11 sequence. Additional nucleotides match the sequence of the genomic *SacI-SacI* fragment, mapping CRABP transcription initiation to the G-residue at position 725 of the Sac880 sequence. During the preparation of this manuscript, Wei *et al.* presented data on the transcriptional mapping of the mouse CRABP gene. From primer extension and RNase protection experiments they conclude that CRABP transcription initiates mainly from the

G-residue 93 bases upstream from the ATG translational initiation codon. However, they also observe longer fragments, presumably corresponding to minor transcriptional initiation sites. The longer cDNAs obtained in the RACE experiment may have originated from one of these latter sites. In any case, we conclude that the 880 bp. *SacI-SacI* fragment must contain the CRABP promoter region.

The sequence of the putative promoter region of mouse CRABP does not exhibit any TATA box homology and is rich in GC. This is in agreement with the results presented by Shubeita *et al.* who have determined the 5' genomic sequence of bovine CRABP. However, no extensive sequence homology is observed between the mouse and bovine CRABP upstream regions. Shubeita *et al.* claim the presence of two putative Sp1 binding sites and three potential cAMP-responsive elements. In the mouse CRABP promoter region, several copies of the Sp1 binding site consensus sequence GGGCGG or CCGCCC (Kadonaga *et al.*, 1986) are present, as indicated in Figure 1.

To see if the *SacI-SacI* CRABP genomic fragment contains a functional promoter, it was linked to the coding sequences of a CAT gene. However, transfection of this reporter gene construct pSaCAT to MES-1 cells failed to produce detectable levels of CAT activity. The apparent lack of CAT expression could be due to several causes, the most likely being that additional regulatory elements are required for activation of the CRABP promoter. Alternatively, pSaCAT would not contain CRABP promoter sequences after all. Also, the presence of the translation initiation site and part of the coding region of the CRABP gene in pSaCAT may interfere with efficient transcription of the linked CAT sequences.

The CRABP promoter-CAT reporter gene constructs pC1000-CAT and pC1000-CAT-Enh were generated to resolve this issue. The genomic fragment contained within these plasmids has its 3' end at the location of the CRABP transcriptional initiation site, and extends approximately 300 bp. further upstream than the *SacI-SacI* fragment. Transfection of pC1000-CAT-Enh into MES-1 cells resulted in a clear stimulation of CAT expression as compared to the vector plasmid pCAT-Enhancer, which demonstrates that the CRABP genomic fragment indeed contains promoter activity. The fact that transfection of pC1000-CAT does not yield any CAT activity indicates that this promoter by itself is insufficient to drive expression of the CAT gene, but apparently requires attendance of enhancer sequences. In cells exhibiting a high level of CRABP expression, the position of such an enhancer would be marked by the occurrence of a nuclease-hypersensitive site in the chromatin. Using a CRABP cDNA fragment containing first and second exon sequences as a probe, we failed to detect DNaseI-hypersensitive sites in MES-1 cells. This would imply that the enhancer sequences are located further upstream, or in the last two introns or downstream of the CRABP gene.

Our experiments show that transcriptional control of the CRABP gene involves *cis*-acting regulatory elements. Additional experiments are required to identify the sequence elements that are necessary and sufficient to drive expression of the CRABP gene in MES-1 cells. It is tempting to speculate that such elements will also direct the spatio-temporally specified expression pattern exhibited by the CRABP gene during embryogenesis.

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Chapter VII

ISOLATION OF HUMAN CELLULAR RETINOIC ACID-BINDING PROTEIN (CRABP) GENE SEQUENCES AND THEIR LOCALISATION ON HUMAN CHROMOSOME 15 RELATIVE TO THE ACUTE PROMYELOCYTIC LEUKEMIA- ASSOCIATED BREAKPOINT.

This chapter has been adapted from:

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Human Genetics, in press

Abstract

We have isolated and sequenced a human genomic clone containing part of the cellular retinoic acid-binding protein (CRABP) gene. By using a panel of somatic cell hybrids, the gene could be assigned to human chromosome 15. A possible involvement of the CRABP gene in translocation (15;17)(q22;q11) positive acute promyelocytic leukemia (APL) was investigated. Although transposition of the CRABP gene to chromosome 17 could be demonstrated, we did not observe gross rearrangements in a series of primary APL patients, nor in the acute myeloblastic leukemia cell line HL-60. Thus, the observed lack of CRABP expression in these leukemic cells does not seem to result from disruption of its gene. CRABP maps to the region 15q22-qter.

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Introduction

Retinoic acid (RA), a biologically active metabolite of retinol (Vitamin A), plays an important role in various growth and differentiation processes both during embryonic development and adult life (Lotan, 1980; Omori and Chytil, 1982; Chytil, 1984; Oikarinen *et al.*, 1985). RA has also been reported to induce *in vitro* differentiation of cancerous cells including human and murine embryonal carcinoma cells (Strickland and Mahdavi, 1978; Andrews *et al.*, 1986), the acute myeloblastic leukemia cell line HL-60, the monoblastic leukemia cell line U-937 (Breitman *et al.*, 1980; Hozumi, 1982), and blasts from acute myeloid leukemia (AML) patients in primary culture (Breitman *et al.*, 1981; Honma *et al.*, 1983). Consequently, RA has been suggested as a potent agent for treatment of AML, in particular acute promyelocytic leukemia (APL) (Breitman *et al.*, 1981; Meng-er *et al.*, 1988; Hassan and Rees, 1990).

The molecular mechanisms underlying RA action are currently subject to intensive investigations. Recent work indicates that RA acts as an endogenous signal molecule that regulates gene expression via nuclear receptors (RARs) in a dose-dependent way (Edwards and McBurney, 1983; Evans, 1988). The discovery of an RA gradient in the developing embryo by Thaller and Eichele (1987) lends support to the idea that morphogenetic processes are mediated by differential RA concentrations. Besides the nuclear receptors, RA-mediated signal transduction also seems to involve the cellular RA-binding protein (CRABP). The spatial and temporal expression of the CRABP gene appears to be strictly regulated during embryonic development. (Vaessen *et al.*, 1989, 1990; Maden *et al.*, 1988, 1989; Perez-Castro *et al.*, 1989), and it has been suggested that CRABP has a function in the formation of RA gradients across various developing tissues (Maden *et al.*, 1989).

Here, we report the isolation of the human homolog of the CRABP gene and its localisation on chromosome 15. A possible involvement of CRABP in the APL-specific translocation (15;17)(q22q11) (Larson *et al.*, 1984) was investigated. Primary APL cells as well as the acute myeloblastic leukemia-derived cell line HL-60 seem to be deficient for CRABP, although RA may induce differentiation of both cell types (Douer and Koeffler, 1982; Allegretto *et al.*, 1983; Jetten *et al.*, 1987; Largman *et al.*, 1989). Using our newly isolated human CRABP probe, we studied CRABP mRNA expression in HL-60 cells prior to and after induction of differentiation.

Materials and Methods

Cells and culture conditions

The panel of human-rodent hybrid cell lines used for the chromosomal localization of the CRABP gene has been described before (Geurts van Kessel *et al.*, 1983), as well as the t(15;17)-derived cell hybrids C73/PJT2A1 and P12.3B6 (Sheer *et al.*, 1983; van Tuinen *et al.*, 1987). The hybrid and parental cells were grown in F10 or DMEM medium according to standard procedures. Chromosome studies were carried out using GTG-banding or R-banding after heat denaturation. The same batches of cells were used for chromosome and DNA analyses.

HL-60 cells were cultured in RPMI-1640 medium supplemented with fetal calf serum (10%), glutamin (2 mM), penicillin (100 u/ml), and streptomycin (100 µg/ml). After addition of either 10⁻⁶ M all-*trans*-RA (from a 3 mg/ml stock in dimethylsulfoxide (DMSO)), or 1% DMSO, the cells were allowed to differentiate for 6 days (Breitman *et al.*, 1980). The medium was refreshed twice during this period. As estimated from May Grünwald-Giemsa stained cytospin preparations, this procedure resulted in differentiation of more than 70% of the cells into granulocytes and monocytes, respectively.

Origin of the human CRABP probe

Screening of the human genomic library CML0 (kindly provided by Dr. G. Grosveld) with mouse and chicken CRABP probes (Vaessen *et al.*, 1989, 1990) yielded several CRABP-positive clones. A repeat-free human *Xho*II fragment of 700 bp. was subcloned into the *Bam*HI site of pTZ18R. The nucleotide sequence of this fragment was determined via the dideoxy chain termination method (Sanger *et al.*, 1977).

Southern blot analysis

DNA isolations were performed according to standard procedures (Jeffreys and Flavell, 1977). Following cleavage with restriction enzymes, DNA fragments were separated by agarose gel electrophoresis, and transferred to Genescreen (NEN) filters (Southern, 1975). ³²P-labelled DNA probes were obtained using random priming as described by Feinberg and Vogelstein (1983). Hybridizations were carried out overnight at 65°C in the presence of 0.5 M phosphate, 7% SDS, and 1mM EDTA. Filters were washed at 65°C: 3 x 5 min. in 40 mM phosphate, 0.1% SDS, and 30 min. in 10 mM phosphate, 0.1% SDS.

Northern blot analysis

Total RNA was extracted from cell lines using the LiCl/Urea method described by Auffray and Rougeon (1980), electrophoresed on 1% agarose gels in the presence of formaldehyde, and transferred to Genescreen filters (Maniatis *et al.*, 1982). Prior to electrophoresis, ethidium bromide was added to the RNA samples in order to allow visualisation of the ribosomal RNAs in the gel. Probe labelling, hybridization, and washing conditions were essentially as described above, but 10% dextran sulphate was included in the hybridization buffer.

Results and Discussion

A 700 bp. repeat-free XhoII fragment of the human CRABP gene was cloned into the *Bam*HI site of pTZ18R to yield HuCRABP-X700, and its nucleotide sequence was determined. As illustrated by Figure 1, comparison of the sequence with that of murine CRABP cDNA revealed a homology of 92% in a stretch of DNA comprising nucleotides 380 to 464 of the mouse sequence (numbering according to Vaessen *et al.*, 1989). Transcriptional mapping of the murine and bovine CRABP genes has demonstrated that nucleotide 464 represents the third exon/intron boundary (Shubeita *et al.*, 1987; Wei *et al.*, 1990). The abrupt ending of sequence homology at this position indicates that this exon/intron boundary is apparently conserved in the human genome as well.

For the chromosomal localisation of the human CRABP gene, we used a panel of 19 well-defined human-rodent somatic cell hybrids (Geurts van Kessel *et al.*, 1983). High molecular weight DNA extracted from these cells, and from control hamster, mouse, and human cells. Southern blot analysis of *Bam*HI-digested DNAs was carried out using HuCRABP-X700 as a probe in hybridization. As shown in Figure 2, this probe hybridizes to a human *Bam*HI fragment of approximately 7 kb. No hybridization was observed with mouse and hamster DNA under these high-stringency conditions. Table I gives an overview of the chromosome/gene concordances as based on the presence or absence of the human CRABP-specific band in the panel of somatic cell hybrids. A high concordance score of 95% was observed for chromosome 15, while concordance scores for other human chromosomes were invariably lower. The one discordant clone had retained chromosome 15 only in a low percentage ($\pm 10\%$) of cells. Therefore, we conclude that the CRABP gene resides on human chromosome 15. This localization is not in agreement with a previous report assigning CRABP on human chromosome 3 (Nilsson *et al.*, 1988). As these investigators used a bovine cDNA probe and low stringency conditions of hybridization, they were probably deluded by cross-hybridization

MoT-CA11 CAGTCTCTCTGCAACGCAGCGTGCACCCGCTCCGCAGCAGAGTGGGTGCCTGCCCTGC 60

MoT-CA11 CTTGCGCCCGCGCTACAGCCAACCCACTGCCACCATGCCCACTTCGCCGGTACCTGG 120
MetProAsnPheAlaGlyThrTrp
↓

MoT-CA11 LysMetArgSerSerGluAsnPheAspGluLeuLeuLysAlaLeuGlyValAsnAlaMet 180
AAGATGCGCAGCAGCGAGAATTCGACGAGCTCCTCAAGCGTGGGTGTGAACGCCATG

MoT-CA11 LeuArgLysValAlaValAlaAlaAlaSerLysProHisValGluIleArgGlnAspGly 240
CTGAGGAAGGTGGCCGTGGCGCTGCGTCTAAGCCGCACGTGGAGATCCGCCAAGACGGG

MoT-CA11 AspGlnPheTyrIleLysThrSerThrThrValArgThrThrGluIleAsnPheLysVal 300
GATCAGTTCTACATCAAGACATCCACTACTGTGCGCACCCACGGAGATCAACTTCAAGGTC

MoT-CA11 GlyGluGlyPheGluGluGluThrValAspGlyArgLysCysArgSerLeuProThrTrp 360
GGAGAGGGCTTCGAGGAGGACAGTGGACGGACGCAATGCAGGAGTTTACCCACGTGG

MoT-CA11 GluAsnGluAsnLysIleHisCysThrGlnThrLeuLeuGluGlyAspGlyProLysThr 420
GAGAATGAGAACAAGATTCACTGCACACAGACACTTCTTGAGGGGGATGGCCCTAAAAC

HC-X700 CACTGCACGCAAACTCTTCTGAAGGGGACGGCCCCAAAACC 42

MoT-CA11 TyrTrpThrArgGluLeuAlaAsnAspGluLeuIleLeuThrPheGlyAlaAspAspVal 480
TACTGGACCCGAGAGCTGGCCAACGATGAGCTAATCCTGACATTTGGCGCGGATGATGTG

HC-X700 TACTGGACCCGTGAGCTGGCCAACGATGAACCTTATCCTGGTAGGAACCTTGACCCTGAAA 102

MoT-CA11 ValCysThrArgIleTyrValArgGlu 540
GTGTGCACAAGAATTTATGTCCGGGAGTAAAGGTGGCCAGCTTGTTCCTGCTTCATGACC

HC-X700 TAATCTTGAAGTTCCCCCAGAG 125

MoT-CA11 GGATGCGAGTCCCTCGAGGATATGCCGTGGCCCCACACTGCCAGTGGGTCTTTACTCC 600

MoT-CA11 ACACACCTCTCCCCATGAATATTAGGCAACCCATTTCCCATGACATGTTGTAGTG 660

MoT-CA11 TCCTCCCTCAGGCTCTTGTTCCTTGTGTACCCCTGGTTGGCATTGTCATGATTGTAC 720

MoT-CA11 CAGTCATTAACCTGGTGGCTGCAAAAAAAAAA

Figure 1

Sequence comparison of *HuCRABP-X700* and mouse *CRABP* cDNA. The *CRABP*-homologous part of the human clone is shown underneath the nucleotide sequence of mouse *CRABP* cDNA clone *MoT-CA11*, with dashes marking identical nucleotides. Presumptive exon/intron boundaries are indicated by arrows (↓).

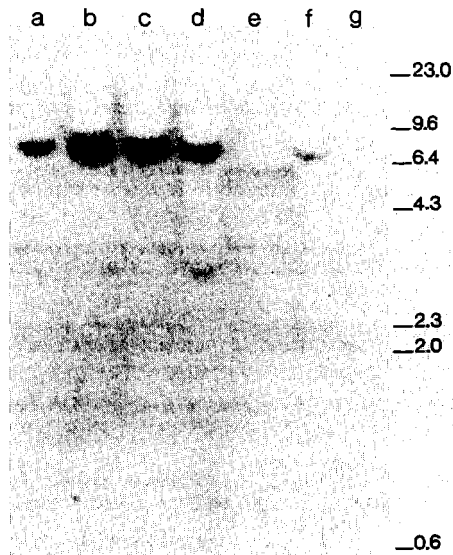


Figure 2

Hybridization of the human CRABP probe to BamHI-digested DNAs isolated from (a) normal human fibroblasts; (b) myeloblastic leukemia cell line HL-60; (c) human teratocarcinoma-derived cell line NT2 clone D1; (d) chromosome 15-only somatic cell hybrid HORL-1; (e) translocation derivative 15/17-positive hybrid C73/PJT2A1; (f) translocation derivative 17/15-positive hybrid P12.3B6; (g) control mouse cells. The molecular weight indications are deduced from HindIII-digested bacteriophage lambda DNA that was co-electrophoresed on the same gel.

with a CRABP-related gene on human chromosome 3.

Hybrid cell lines obtained by fusion of cells from two t(15;17)-positive APL patients were used to establish the localisation of the CRABP gene relative to the APL-associated t(15;17)(q22;q11) breakpoint on chromosome 15. DNA isolated from hybrids containing either the 15/17 or the 17/15 translocation derivative in the absence of a normal chromosome 15, was subjected to Southern blot analysis. Normal human

chromosome	chromosome / CRABP			+/+	% discordance
	+/+	+/-	-/+		
1	4	5	6	4	58
2	2	2	8	7	53
3	5	4	5	5	47
4	6	3	4	6	37
5	5	5	5	4	43
6	5	4	5	5	47
7	5	2	5	7	37
8	7	6	3	3	47
9	6	6	4	3	53
10	3	4	7	5	58
11	4	5	6	4	58
12	7	2	3	7	26
13	7	5	3	4	42
14	8	5	2	4	37
15	10	*1	0	8	5
16	7	6	3	3	47
17	9	5	1	4	32
18	4	5	6	4	58
19	7	5	3	4	42
20	9	7	1	2	42
21	7	7	3	2	53
22	7	6	3	3	77
X	6	2	4	7	32

*: this hybrid clone contains chromosome 15 in a low percentage (10%) of cells.

Table 1

Correlation between the presence or absence of human chromosomes and the CRABP gene in 19 human-rodent somatic cell hybrids.

fibroblasts, the acute myeloblastic leukemia cell line HL-60, and a human chromosome 15-only hybrid (HORL-1; Spurr, unpublished) were also included in this analysis. As can be seen in Figure 2, HORL-1 is CRABP-positive, confirming our assignment of the gene to human chromosome 15. The faint extra band of approximately 2.7 kb. that is detected in this hybrid is probably due to a chromosomal rearrangement present in a minority of the cells. The hybrid C73/PJT2A1, which contains the 15/17 derivative of the t(15;17)

translocation, is negative, whereas P12.3B6, containing the 17/15 derivative, is positive for CRABP. We conclude that the t(15;17) translocation that occurred in these APL patients has resulted in transposition of the CRABP gene from chromosome 15 to chromosome 17.

In the APL-derived hybrids, HuCRABP-X700 hybridizes to an *Bam*HI fragment of similar size as detected in normal human fibroblasts. In DNA isolated from HL-60 cells no hybridizing fragments of aberrant size were observed either. As similar results were obtained with *Eco*RI cleaved DNAs, it appears that no gross rearrangements have occurred within the CRABP sequence that is recognized by HuCRABP-X700. DNA analysis of another eight t(15;17)-positive APL patients failed to detect abnormal CRABP fragments, providing additional evidence that the APL-specific t(15;17) breakpoint does not occur within restriction fragments hybridizing to our CRABP probe.

During the preparation of this manuscript it was reported by two independent groups that the APL breakpoint at chromosome 17 is located within the gene encoding retinoic acid receptor α (RAR α) (Borrow *et al.*, 1990; De Thé *et al.*, 1990). The translocation appears to result in formation of a chimeric transcript where the 5' part of the RAR α transcript has been replaced by a novel sequence, encoded by the gene on chromosome 15. The fusion protein would retain the DNA- and ligand-binding domains of RAR α , but the transcription-activating function would be replaced, potentially changing the profile of target gene specificity. However, a possible involvement of the mutant RAR α in leukemogenesis remains to be investigated.

It has been reported that the myeloblastic leukemia-derived cell line HL-60 does not contain any CRABP (Douer and Koeffler, 1982; Jetten *et al.*, 1987; Largman *et al.*, 1989). In order to extend these studies to the mRNA level, we have performed Northern blot analyses. RNA was extracted from exponentially growing HL-60 cells as well as from HL-60 cells that were induced to differentiate. Granulocytic differentiation was brought about by treatment with 10^{-6} M RA; monocytic differentiation was induced by exposure to 1% DMSO. Using HuCRABP-X700 as a probe in hybridization, we did not detect CRABP transcripts in any of the HL-60-derived RNA samples (not shown). Since the CRABP gene does not seem to be grossly disrupted in these cells, the observed lack of CRABP expression may find its cause in transcriptional repression. It will be of interest to investigate a conceivable relationship to the presence of the translocated RAR α , and to evaluate a possible role of CRABP in (aberrant) myeloid differentiation processes and the development of APL.

Acknowledgements

The authors thank Dr. D. Bootsma and Dr. H.H. Ropers for advice and support, Dr. D. Sheer and Dr. E. Solomon for providing hybrid cell DNAs, and Dr. R. Berger for providing DNAs from primary APL patients. We also acknowledge Dr. G. Grosveld for gift of the human genomic library. This work was supported by the Netherlands Cancer Society (Koninkrijk Wilhelmina Fonds) and the Netherlands Organization for Scientific Research (NWO-BION).

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Chapter VIII

GENERAL DISCUSSION

1. Identification of genes that are transcriptionally induced upon differentiation of an embryonal carcinoma cell line

It is generally assumed that the process of embryonic development involves differential expression of specific sets of genes (Morgan, 1934). Such differentially expressed genes obviously include many household genes, coding for a wide variety of enzymes and structural proteins, but the developmental control genes themselves will also exhibit specific patterns of expression. Indeed many classes of transcriptional regulators have been isolated and shown to play important roles in embryogenesis (Dressler and Gruss, 1988). The work described in this thesis was aimed at the isolation and characterization of gene sequences that are specifically induced upon differentiation of embryonal carcinoma (EC) cells. Doing this, we hoped to acquire gene markers that are specific for particular lines of differentiation, and possibly gain insight in the molecular processes taking place during embryonic development.

We used the murine P19 EC cell system as a model for the *in vitro* study of cellular differentiation. This particular EC cell line can be induced to form various types of differentiated derivatives, representing all three embryonic germ layers (McBurney *et al.*, 1982). Clonal cell lines have been obtained of endodermal, ectodermal and mesodermal P19 derivatives (Mummery *et al.*, 1985; 1986), which allowed us to compare undifferentiated and differentiated cells that are alike in their genetic background. Two reasons impelled us to concentrate on the mesodermal line of differentiation, represented by the cell line MES-1. Firstly, this line of differentiation clearly contributes to the formation of structures of the embryo itself rather than extra-embryonic tissues. Secondly, MES-1 was the first EC derived cell line that exhibited several general properties of mesodermal cells (Mummery *et al.*, 1986). Therefore, use of the MES-1 cell line enabled us to gain information on a line of EC cell differentiation that was little characterized at the time.

For the isolation of gene sequences that are abundantly expressed in MES-1 cells as compared to undifferentiated P19 EC cells, we performed differential screening of a MES-1 cDNA library. This approach resulted in the isolation of nine independent cDNA clones. After determination of the nucleotide sequences, database comparison revealed that we had selected cDNA clones corresponding to the following genes:

- vimentin
- placental Ca²⁺-binding protein (pCaBP)
- β galactoside-binding lectin
- cellular retinoic acid-binding protein (CRABP)

The cDNA cloning of vimentin, an intermediate filament typically found in cells of mesenchymal origin, and of three proteins which have been implicated in cellular differentiation and proliferation processes, proves that our experimental approach did indeed serve the original purpose.

2. Implications for the nature of MES-1 cells in terms of normal embryonic development

The identification of gene sequences that are induced upon differentiation of P19 EC into mesoderm-like derivatives, allows a reconsideration of the validity of this model system for differentiation events taking place during embryonal development. The MES-1 cell line exhibits several general mesodermal characteristics, including expression of receptors for and response to PDGF, and it has been suggested that MES-1 represents an EC-derived myoblast (Mummery *et al.* 1986). However, the exact nature of MES-1 cells in terms of the cell type they might represent during normal development has not as yet been clearly established. In this respect, our demonstration that vimentin, placental Ca²⁺-binding protein, and β galactoside-binding lectin are transcriptionally induced in MES-1 is not particularly informative, since expression of these proteins is not known to be restricted to specific embryonal cells. However, the high CRABP expression observed in MES-1 cells is much more indicative. The CRABP gene exhibits a spatio-temporally specified expression pattern during embryogenesis, with high expression occurring in parts of the central and peripheral nervous system, the craniofacial and visceral arch mesenchyme, and the developing limb bud (Maden *et al.*, 1988; 1989; 1990; Vaessen *et al.*, 1989; 1990; Perez-Castro *et al.*, 1989; Dollé *et al.*, 1989). Thus, the *in vivo* expression of CRABP does not correlate with the presumed myoblast-like character of MES-1. The morphology of the MES-1 cells, coupled to the high level of CRABP transcripts rather suggests that MES-1 is a representative of either the neural crest-derived craniofacial or visceral arch mesenchyme, or the chondrogenic cells of the limb bud.

In relation to this, it may be significant that even the undifferentiated P19 EC cells express several markers that are suggestive for a certain commitment to the neuronal line of differentiation. Most notable in this respect is the fact that P19 EC cells contain a high level of neurofilament light chain (NF-L) mRNA (An Langeveld,

unpublished results). P19 EC has been noted for its RA-induced capacity to differentiate in a manner closely resembling that of embryonic brain tissue; that is, cells develop into neurons, glia, and fibroblast-like cells (Jones-Villeneuve *et al.*, 1983). Differentiation of P19 EC into a cell type that bears resemblance to neural crest-derived mesenchyme would be in line with this presumed neurogenic character. However, it is not clear how DMSO-induced differentiation of P19 EC into skeletal and cardiac muscle fits into this picture, unless we assume that P19 represents a stem cell with myogenic as well as neurogenic properties. Indeed, developing nervous tissue and developing skeletal muscle have been reported to share other unexpected properties. The intermediate filament nestin, which is regarded as a general marker for CNS stem cells, is also expressed at low levels in developing skeletal muscle (Lendahl *et al.*, 1990). Furthermore, medulloblastoma tumors, presumably of single cell origin, produce cells with characteristics of nerve and muscle (Rubinstein, 1985).

Multipotency of EC cells is illustrated by their ability to contribute to the formation of chimeric mice. Injection of karyotypically normal P19 EC cells into mouse blastocysts has been reported to result in efficient production of chimeric mice with a wide variety of apparently normal tissues colonized by the EC cells. However, most chimaeras were morphologically abnormal. In view of the above it may be significant that abnormal development mostly occurred in the brain and head region (Rossant and McBurney, 1982). Thus, after reintroduction into mouse embryos, P19 EC cells do not behave completely like normal embryonic cells, showing incomplete regulation by the embryonic environment.

Taken together, the P19 EC cell system has proven itself to be a useful model for the *in vitro* study of cellular differentiation events. However, it is not feasible to define either the undifferentiated EC cells or the differentiated derivatives as true representants of a particular cell type present during normal embryonic development. Therefore, extrapolation of expression data obtained using the P19 EC cell system to differentiation processes occurring *in vivo* should not be taken as a matter of course.

3. The biological function of CRABP

One of the clones that was isolated via differential screening of a MES-1 cDNA library was identified as encoding the cellular retinoic acid binding protein (CRABP). RNA blot analysis confirmed that the CRABP gene is abundantly expressed in MES-1 cells as compared to undifferentiated P19 EC cells. Investigation of the other P19 derivatives revealed that a low level of CRABP mRNA is expressed in EPI-7 cells, whereas no CRABP transcripts could be detected in END-2 cells. It should be noted that both EPI-7

and END-2 were obtained after treatment of P19 EC cells with RA. Although others have reported that CRABP expression is elevated in P19 or F9 EC cells after induction with RA (Eriksson *et al.*, 1986; Wei *et al.*, 1987; Stoner and Gudas, 1989), we did not observe a strong increase in the level of CRABP transcripts after RA-treatment of P19 EC cells. We propose that induction of the CRABP gene is not mechanistically involved in RA-mediated differentiation of EC cells, but simply occurs as a consequence of differentiation to a particular cell type.

However, the highly specific binding affinity for RA unquestionably indicates that CRABP expression must in some way play a role in the RA-mediated signal transduction pathway. Strong evidence for a vital biological function of CRABP comes from the highly specific expression pattern during embryogenesis. A graded distribution of CRABP was demonstrated in the limb bud (Maden *et al.*, 1988; Dollé *et al.*, 1989), and a specific expression pattern also occurs in the central nervous system (Vaessen *et al.*, 1989; Maden *et al.*, 1989; 1990; Perez-Castro *et al.*, 1989). Other embryonic tissues exhibiting high CRABP expression - the craniofacial mesenchyme and the visceral arch area (Vaessen *et al.*, 1989; 1990) - have been known to be affected by the teratogenic action of RA. Thus, the *in vivo* expression pattern of CRABP exhibits a striking correlation to tissues that are often affected by fetal exposure to excess RA, which suggests that high expression of CRABP in a certain tissue reflects a particular sensitivity to RA. This notion is reinforced by the observation that the biological activity of numerous synthetic retinoids correlates remarkably well with their binding affinity for CRABP (Jetten and Jetten, 1979; Lotan, 1980; Sani *et al.*, 1984; Keeble and Maden, 1986).

The biological function of CRABP is not quite understood. The fact that binding of RA to nuclear extracts could be observed only in extracts prepared from cells that were preincubated with RA was formerly taken to indicate that binding of RA to a cytoplasmic binding protein - CRABP - is obligatory for transfer of RA to the nucleus (Jetten and Jetten, 1979). The discovery of a family of nuclear RA receptors, at least one of which (RAR β) is induced by RA treatment (De Thé *et al.*, 1989; Song and Siu, 1989; Hu and Gudas, 1990), provides a new explanation for these experiments. More direct proof against a function of CRABP as an RA-shuttle protein is given by the *in vivo* expression pattern. In the limb bud, CRABP transcripts have a graded distribution (Maden *et al.*, 1988). Interestingly, this CRABP gradient is of opposite polarity to the concentration gradient of RA itself (Thaller and Eichele, 1987). Hence, Maden *et al.* suggest that CRABP is involved in lowering the effective concentration of unbound RA, thus steepening the morphogenetic RA gradient. Our data concerning the cellular response to RA in EC cells containing different levels of CRABP (see Chapter V) are in line with the idea that CRABP has a function in the absorption of RA, preventing RA from reaching the nuclear receptor sites.

4. The role of RA in embryonic development

For a better understanding of the function of CRABP, a more detailed perception of the biological effects of RA seems to be essential.

RA has profound effects on a variety of biological processes. The biological importance of RA in cellular differentiation and proliferation processes, and in maintenance of the differentiated state of many if not all epithelia has long been recognized (reviewed by Dicken, 1984). RA is also known as a potent teratogen, causing primarily heart defects, craniofacial malformations, and central nervous system abnormalities (Lammer *et al.*, 1985). In addition, RA has morphogenetic activity in various developmental processes, including primary axis formation (Durstun *et al.*, 1989; Mitrani and Shimoni, 1989) and organization of the skeletal pattern in the developing and regenerating limb (Tickle *et al.*, 1982; Eichele, 1989; Tickle *et al.*, 1989). The actual demonstration of differential levels of endogenous RA across the anteroposterior axis of the limb bud (Thaller and Eichele, 1987) suggests that RA may indeed be an important natural morphogen.

Several mechanisms have been proposed that could account for the dramatic effects of RA on embryonic development. *in vitro* experiments have made it clear that RA is important for cellular differentiation and proliferation. Extrapolation of these observations to the *in vivo* situation may be justified. Axis formation constitutes a very complex process in which different types of cells are being induced to take alternative differentiation pathways. Hence, a disturbance of normal differentiation, brought about by excess RA, may well affect embryonic pattern formation. Abnormal differentiation may also in part explain the teratogenic effects of RA. The etiology of RA-induced craniofacial malformations is well-documented (Wiley *et al.*, 1983; Goulding and Pratt, 1986; Webster *et al.*, 1986; Wedden, 1987; Sulik *et al.*, 1988; Abbott *et al.*, 1989). Exposure of the palatal shelves in mouse embryos affects proliferation of the mesenchymal component, and may invoke two different programmes of inappropriate differentiation in the medial epithelial component (Abbott *et al.*, 1989). After exposure to RA on day 10, small palatal shelves form which fail to make medial contact. The medial cells differentiate into a stratified, squamous, keratinizing epithelium, which further prevents fusion of the shelves. Exposure on day 12 does not cause mesenchymal growth retardation, but may result in cleft palate as an effect of differentiation of the medial cells into a pseudostratified, ciliated, secretory epithelium. Apparently the effects of RA vary with the stage of development exposed.

A different explanation accounting for RA-induced birth defects is that RA is cytotoxic for particular cells, and may have a function in programmed cell death.

Exposure of early stage embryos to RA (chicken day 2; late neurula) results in a high mortality rate (Jelinek and Kistler, 1981). It has been demonstrated that the pathogenesis of RA-induced craniofacial malformations involves excessive cell death in regions of physiological cell death (Sulik *et al.*, 1988; Alles and Sulik, 1989). Similarly, RA-induced spina bifida seems to entail excessive cell death, predominantly in the mesenchyme associated with the primitive streak (Alles and Sulik, 1990). However, after exposure of day 10 or 12 mouse embryos resulting in cleft palate, no excessive or premature cell death of the medial cells was observed (Abbott *et al.*, 1989).

RA-induced fetal malformations predominantly affect tissues that have in common a mesenchymal component which is derived in part from the cranial neural crest. This observation has led to the suggestion of a more specific mechanism accounting for the teratogenic effects of RA, namely that RA specifically interferes with normal neural crest cell migration. This idea is reinforced by *in vitro* experiments showing that vitamin A and RA interfere with neural crest cell-substratum adhesion (Thorogood *et al.*, 1982; Smith-Thomas *et al.*, 1987). In fact, if embryos are exposed to RA at a stage where cranial neural crest cells are migrating, cleft palate induction indeed appears to be a consequence of a failure in neural crest cell migration. Goulding and Pratt (1986) as well as Webster *et al.* (1986) have proposed a direct cytopathic effect with alterations in cell morphology and reduced neural crest cell numbers resulting from either cell death or decreased proliferation. Alternatively, it has been proposed that interference with migration and localization of neural crest cells may account for the observed malformation syndrome (Hassell *et al.*, 1977; Keith, 1977; Kirby and Bockman, 1984; Thorogood *et al.*, 1982). The correlation between susceptibility to RA of an embryonic tissue and the occurrence of neural crest cell derivatives is too strong to be coincidental. However, it should be kept in mind that RA may still induce malformations in embryos well past the stage of neural crest cell migration.

A question that none of the proposed mechanisms can fully explain is why some tissues are susceptible to RA treatment, and others remain largely unaffected. For example, only the neural crest anterior to the 5th pair of somites is capable of mesenchymal differentiation and contributes to normal craniofacial and cardiovascular development (LeLievre and LeDouarin, 1975; Noden, 1978; Philips *et al.*, 1987). It is not clear how RA could specifically affect this subpopulation of neural crest cells. Another point of discussion is whether the different biological effects of RA can be attributed to one and the same mechanism of action. For instance, it is not clear whether RA-induced fetal malformations can be related to disturbance of normal pattern formation or must be ascribed to different effects of RA. It should be noted that the morphogenetic effects of RA on the limb produce structures that have normal morphology but that arise at an abnormal position on the developmental axis. In this respect they are distinct from the

teratogenic effects of RA, which give rise to underdeveloped structures at normal positions.

It has been proposed that the effect of RA is biphasic, with general cytotoxic effects prevailing following earliest exposure, and specific malformations produced with later exposures (Jelinek and Kistler, 1981). In the first phase, during formation of organ anlagen, RA would act without specificity upon poorly differentiated cells. The second phase would be specific, and dependent on the cellular differentiation program. It seems likely that RA may elicit different responses in different cell types, all depending on the state of differentiation and the cellular context. The developmental fate of certain embryonic cells is undoubtedly influenced by cell-to-cell interactions, components of the extracellular matrix, and growth factors present in the embryo. It has been suggested that RA is used as a powerful switch, starting either of a number of developmental processes (Brockes, 1990).

5. Molecular mechanisms of RA action

Although many questions concerning the biological effects of RA remain, at least some understanding has been reached on the molecular mechanisms via which RA mediates these effects. The discovery of a family of nuclear RA receptors which have the characteristics of transcription factors (Petkovich *et al.*, 1987; Giguère *et al.*, 1987; Brand *et al.*, 1988; Krust *et al.*, 1989; Ragsdale *et al.*, 1989; Evans, 1988) strongly supports the idea that RA exerts transcriptional control of specific gene sequences. It is assumed that binding of RA activates the RARs, which subsequently effect transcription of specific genes. Thus, RA-induced fetal malformations would be a result of aberrant gene expression. In relation to this, it is noteworthy that embryos do not develop normally in the absence of retinoids (Kalter and Warkany, 1959). The malformations observed in these RA-deficient embryos seem to affect the same tissues as those susceptible to the adverse effects of excess RA. It must be assumed that regular expression of certain developmental control genes is required for a normal development of these tissues, and that endogenous RA concentrations are critical for the transcriptional control.

For a better understanding of the molecular processes concerned with embryonic pattern formation, it will be of prime importance to identify the genes that are the target of RA action. In this respect, the homeobox-containing gene family, members of which are assumed to act as transcriptional regulators, deserves special attention. All murine homeobox genes described so far show some tissue or region specificity in the spatial distribution of their expression during embryogenesis and it is thought that they participate in pattern formation and segmentation of the mammalian embryo (reviewed

by Dressler and Gruss, 1988). *in vitro* experiments have demonstrated that the homeobox containing genes are transcriptionally up-regulated by RA in a dose-dependent manner (Colberg-Poley *et al.*, 1985a, b; Breier *et al.*, 1986; Deschamps *et al.*, 1987; Mavilio *et al.*, 1988; Simeone *et al.*, 1990). If *in vivo* transcription of the homeobox genes is similarly dependent on RA, a low RA concentration could be instrumental in prohibiting homeobox gene expression in a particular embryonic region during critical stages of normal development. In this way, RA could influence expression of a very complex network of regulatory genes. Thus, even developmental processes as complex as axis formation could be controlled by differential RA concentrations within the embryo.

6. Regulation of RA concentrations within the embryo; a role for CRABP?

An implication of the hypothesis outlined above is that cells would be extremely vulnerable to abnormal RA concentrations. It is conceivable that the presumed susceptibility to RA-induced expression of developmental control genes is limited to specific stages of differentiation. This would be in line with the observation that RA sensitivity of embryonic processes is clearly stage-dependent (Jelinek and Kistler, 1981; Birnbaum *et al.*, 1989; Abbott *et al.*, 1989; Hart *et al.*, 1990)

Nevertheless, during critical periods of development regulation of RA concentrations within the embryo would be extremely important. The enzymes involved in the metabolism of retinol and retinyl esters could exert a certain amount of control (Siegenthaler *et al.*, 1990), but this would not be sufficient. For one thing, RA is a very small molecule which diffuses easily across the embryo, thus complicating the effectuation of a critical RA concentration. Furthermore, embryonic morphogenetic processes involve multiple cells and tissues at the same time. A mechanism for local fine-tuning of RA concentrations seems to be indispensable.

Based on the results described in this thesis we suggest that this regulation of local RA concentrations could be achieved by differential expression of CRABP. All embryonic tissues exhibiting high CRABP expression appear to be responsive to RA. We propose that a high level of CRABP marks the developmental stage during which the cells are open to the transcriptional control exerted by RA.

The above can be illustrated by a recent report by Balling *et al.* that ectopic expression of Hox1.1 in transgenic mice results in craniofacial abnormalities, a phenotype reminiscent of RA-induced malformations (Balling *et al.*, 1989). Our *in situ* hybridization experiments have demonstrated that the craniofacial mesenchyme contains a high level of CRABP transcripts. In analogy to the limb bud, this high CRABP expression may result in a low concentration of unbound RA in this embryonic region, thus averting

Hox1.1 expression. In case of fetal exposure to excess RA, even a high level of CRABP would not be sufficient to prevent RA from reaching the nucleus, which would result in aberrant Hox1.1 expression. This model would explain why administration of excess RA as well as introduction of Hox1.1 sequences linked to a constitutive, RA-independent promoter result in the same phenotype.

7. Concluding remarks

The discussion on the biological function of CRABP has been further complicated by the recent demonstration that at least two dissimilar forms of CRABP are present in mouse and chicken, exhibiting a distinct tissue distribution in embryonic and adult animals (Bailey and Siu, 1988, 1990; Kitamoto *et al.*, 1988, 1989; Giguère *et al.*, 1990). The two CRABPs, designated CRABP I and CRABP II, share extensive sequence homology, but seem to be encoded by different genes (Giguère *et al.*, 1990). It has been suggested that additional members of this putative family of CRABP-related genes may exist. Provisional binding studies have shown that CRABP II has a specific affinity for RA, but the authors cannot exclude the possibility that another retinoid could be the natural ligand associated with CRABP II (Giguère *et al.*, 1990). In relation to this, the demonstration by Thaller and Eichele that the retinoid 3,4-didehydroretinoic acid (ddRA) is present in significant quantities in the chick embryo and is able to induce digit duplications in the limb bud with potency comparable to that of RA (Thaller and Eichele, 1990), could gain even more importance.

At the moment, it appears that the influence of retinoids on embryonic development depends on a complex line of events, each governed by its own, possibly interconnecting regulation mechanisms. On the first level, the vitamin-A metabolism may give rise to a number of morphogenetically active retinoids. These retinoids may be bound by multiple binding proteins, including at least two types of CRABP. The CRABPs seem to be involved in regulating the access of RA to a family of nuclear RA receptors, which appear to regulate expression of a whole network of developmental control genes. The elucidation of these intricate processes will undoubtedly be a major challenge, occupying scientific interest for many years to come.

But then, of course it was never to be expected that embryonic pattern formation would turn out to be simple.

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Summary

Retinol (Vitamin A) and retinoic acid (RA) are thought to play an important role in cellular differentiation and proliferation processes. RA is a potent teratogen and has been known to cause severe birth defects upon administration during pregnancy. Interestingly, RA also exhibits morphogenetic activities. Local administration of RA to the embryonal chick limb may give rise to digit duplications. Moreover, RA was shown to be present in the developing chick limb bud, forming a concentration gradient across the anteroposterior axis. These results have led to the hypothesis that RA is involved in embryonic pattern formation.

The molecular mechanism of RA action probably entails binding of RA to specific nuclear receptor proteins, several of which have been identified. These RA-receptors exhibit the general characteristics of transcription factors, and are related to the steroid/thyroid hormone receptors. It is generally assumed that interaction of RA with the RA-receptors mediates expression of specific gene sequences, thus altering the properties of the cell.

The cytoplasmic RA-binding protein CRABP seems to play an important role in the RA-signal transduction pathway. *Chapter II* describes how selection of cDNA clones, corresponding to gene sequences that are induced upon differentiation of an embryonal carcinoma cell line, has resulted in the isolation of a CRABP cDNA.

Chapter III deals with expression of CRABP during mouse embryogenesis. Using *in situ* hybridization experiments, high expression of CRABP was shown to be limited to specific embryonal cells, depending on the stage of development. *Chapter VI* further elaborates on the spatio-temporal expression pattern of CRABP, extending studies to the chicken embryo. In both organisms, a high level of CRABP transcripts is detected in specific parts of the central nervous system, the craniofacial mesenchyme, the visceral arch area, and the limb bud. Thus, a striking correlation is observed between high CRABP expression and sensitivity of embryonic tissues to RA.

Remarkably, a high level of CRABP expression is detected in the anterior part of the developing limb, whereas the concentration of RA itself is highest in the posterior part. This finding has led to the idea that CRABP has a function in reducing the local concentration of RA, freely available for the activation of the nuclear RA-receptors. Thus, differential expression of CRABP would be involved in the generation of the putative morphogenetic fields. However, the exact role of CRABP in the RA-mediated signal transduction pathway is not clear. *Chapter V* presents some tissue-culture experiments that were performed to clarify this issue. RA can induce expression of the gene encoding RAR β , one of the RA-receptors. The sensitivity of RAR β expression to

RA-induction was studied in two related cell lines - one containing a high level of CRABP, the other CRABP-deficient. The results of these experiments suggest that a high level of CRABP impedes RA-induced gene activation. This would point to a biological function of CRABP in protecting specific embryonic cells and tissues from aberrant gene induction by RA.

Such an important function would obviously necessitate a strict regulation of the CRABP gene, presumably taking place at the transcriptional level. In *Chapter VI*, the characterization of the regulatory elements that control CRABP expression is initiated. Our results show that the minimal promoter sequences are not sufficient to drive high level transcription, suggesting that spatio-temporally specific expression of the CRABP gene requires the presence of additional regulatory elements.

Chapter VII describes the cloning of the human CRABP gene, and its assignment to chromosome 15. The human promyelocytic leukemia cell line HL-60, which is characterized by the occurrence of a translocation between chromosomes 15 and 17, is CRABP-deficient. However, no gross rearrangements were detected in the CRABP gene. Therefore, the cause of the observed CRABP-deficiency remains obscure.

The experiments presented in this thesis contribute to the understanding of the RA-induced signal transduction pathway and its role in embryogenesis. The results give a good example of how "reverse genetics" - the cloning of gene sequences of unknown function on the basis of the expression patterns, followed by characterization of the gene products - can provide insight in fundamental biological processes.

Samenvatting

Retinol (vitamine A) en retinylzuur (Retinoic Acid, RA) worden verondersteld een belangrijke rol te spelen bij de differentiatie en proliferatie van bepaalde celtypen. RA heeft ook een teratogene werking en kan, na toediening tijdens de zwangerschap, aanleiding geven tot ernstige aangeboren afwijkingen. Bijzonder interessant is de ontdekking dat RA morfogenetische activiteit bezit. Lokale behandeling van een embryonale kippepoot met RA kan aanleiding geven tot duplicatie van elementen. Bovendien is aangetoond dat in de zich ontwikkelende kippepoot endogeen RA aanwezig is, waarbij een concentratiegradient optreedt langs de antero-posterior as. Deze resultaten hebben geleid tot de hypothese dat RA betrokken is bij de embryonale patroonvorming.

Het moleculaire werkingsmechanisme van RA berust vermoedelijk op de binding van RA aan specifieke nucleaire receptoren, waarvan er inmiddels verschillende zijn geïdentificeerd. Deze RA-receptoren bezitten de eigenschappen van transcriptiefactoren, en vertonen verwantschap met de receptoren van steroïde- en thyroïde hormonen. De conclusie lijkt gerechtvaardigd dat RA, via complexvorming met een RA-receptor, aanleiding geeft tot inductie van bepaalde genen, en dat RA langs deze weg de eigenschappen van de betreffende cel beïnvloedt.

Een belangrijke rol in de keten van RA-siginaaloverdracht lijkt te worden ingenomen door het cytoplasmatische RA-bindende eiwit CRABP. *Hoofdstuk II* van dit proefschrift beschrijft hoe de selectie van cDNA kloons, overeenkomend met genen die geïnduceerd worden tijdens differentiatie van een muize embryonaal carcinoma cellijn, onder andere geresulteerd heeft in de isolatie van een cDNA kloon die codeert voor CRABP.

De expressie van CRABP tijdens de embryonale ontwikkeling van de muis is het onderwerp van *Hoofdstuk III*. Hierin wordt met behulp van *in situ* hybridisatie technieken aangetoond dat een hoge expressie van CRABP beperkt is tot bepaalde embryonale cellen en ontwikkelingsstadia. De resultaten voor wat betreft het CRABP expressiepatroon zijn verder uitgebouwd in *Hoofdstuk IV*, met dien verstande dat hier met kippe-embryo's is gewerkt. In beide organismen wordt een hoge CRABP expressie waargenomen in bepaalde delen van het centraal zenuwstelsel, in het craniofaciale mesenchym, in het kieuwbooggebied, en in de pootaanleg. Er blijkt een duidelijke correlatie te bestaan tussen gevoeligheid van bepaalde embryonale weefsels voor RA, en het optreden van een hoge expressie van CRABP.

Opmerkelijk is, dat een hoge CRABP expressie in de zich ontwikkelende poot juist op die plaats optreedt waar een lage concentratie van RA gemeten wordt. Dit heeft

aanleiding gegeven tot de gedachte dat CRABP de concentratie van vrij RA, beschikbaar om de nucleaire RA-receptoren te activeren, verlaagt. Differentiële expressie van CRABP zou dan betrokken zijn bij de vorming van de morfogenetisch actieve RA-concentratiegradient. Het is echter niet duidelijk welke rol CRABP precies speelt in de keten van RA-siginaaloverdracht. *Hoofdstuk V* geeft een beschrijving van weefselkweek-experimenten die werden uitgevoerd met het doel hierin opheldering te verschaffen. RA is in staat om expressie van het gen coderend voor RAR β , één der nucleaire RA-receptoren, te induceren. De gevoeligheid van deze RAR β expressie voor RA-inductie werd vergeleken in twee verwante cellijnen - één met een hoge CRABP expressie, de andere CRABP-deficiënt. De resultaten van dit onderzoek suggereren dat gen-activatie door RA belemmerd wordt door grote hoeveelheden CRABP. In overeenstemming hiermee zou de biologische functie van CRABP in de embryonale ontwikkeling kunnen zijn, om bepaalde cellen en weefsels te beschermen tegen misplaatste gen-inductie onder invloed van RA.

Een dergelijke belangrijke rol van CRABP in de embryogenese vooronderstelt een strikte regulatie van de CRABP gen-expressie. In *Hoofdstuk VI* wordt dan ook een aanzet gegeven tot de identificatie van de regulerende elementen die van belang zijn voor de expressie van het CRABP gen. De resultaten geven aan dat de minimale promotersequentie niet voldoende is om hoge CRABP expressie te bewerkstelligen, en dat bepaalde, nog niet geïdentificeerde, enhancer elementen noodzakelijk zijn voor weefsel-specifieke CRABP expressie.

De isolatie van het humane CRABP gen, en de localisatie op het humane chromosoom 15, is beschreven in *Hoofdstuk VII*. Humane promyelocyttaire leukemie cellen die gekarakteriseerd worden door een translocatie tussen de chromosomen 15 en 17, zijn CRABP deficient. Het CRABP genlocus lijkt echter niet bij de translocatie betrokken te zijn, zodat de oorzaak van de optredende CRABP deficiëntie vooralsnog onopgehelderd blijft.

De experimenten die in dit proefschrift beschreven zijn, dragen bij tot een beter begrip van de door RA geïnduceerde keten van signaaloverdracht en de rol daarvan in de embryonale ontwikkeling. De resultaten van het onderzoek laten duidelijk zien dat de "omgekeerde genetica" - het op basis van het expressiepatroon kloneren van genen van waarvan de functie onbekend is, gevolgd door het karakteriseren van de bijbehorende eiwitproducten - belangrijke inzichten kan verschaffen in fundamentele biologische processen.

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Dankwoord

Dit proefschrift is hoofdzakelijk gebaseerd op de resultaten van het KWF-project IKR 85/2, getiteld "The molecular basis of initiation and progression of human teratocarcinoma". Ik dank mijn promotor, Dick Bootsma, en mijn co-promotor, Ad Geurts van Kessel dat zij mij de gelegenheid hebben geboden dit onderzoek te verrichten. Ik ben hen dankbaar voor het gestelde vertrouwen en voor de vrijheid die zij mij lieten bij de invulling van het project. Ook wil ik hierbij de overige mensen bedanken die meegewerkt hebben aan de het tot stand komen van het project: Ted Splinter, Siegfried de Laat, en Maarten Mulder. Ik ben me bewust dat het verrichte onderzoek niet direct in overeenstemming is met de oorspronkelijke doelstellingen, maar ik meen dat de gevolgd lijn gedictieerd werd door de resultaten.

Veel dank ben ik verschuldigd aan de mensen die betrokken zijn geweest bij de uitvoering van de experimenten. Dat geldt natuurlijk in de eerste plaats de mensen van de Terato-groep: Erika Kootwijk, Wilma Keijzer, An Langeveld, Maarten Mulder, Rijka Beekhuizen, Leonie Rijks, en Erik-Jan Dekker. Echter ook de overige medewerkers van de afdeling Celbiologie en Genetica wil ik hierbij bedanken voor de prettige sfeer van samenwerking en de algemene hulpvaardigheid. Veel steun heb ik met name ondervonden van Gerard Grosveld en Dies Meijer, die altijd bereid waren mee te denken en een oplossing te zoeken voor eventuele problemen. Onontbeerlijk was ook de hulp van Sjozèf van Baal bij alle handelingen die het gebruik van een computer vereisten.

De veelzijdige expertise die aanwezig is binnen de afdeling bleek van grote waarde toen de verkregen resultaten aanleiding gaven om een oorspronkelijk moleculair biologisch project om te buigen in de richting van de ontwikkelingsbiologie. Carel Meijers en Arthur van der Kamp en hun medewerkers hebben mij hierbij fantastisch geholpen. Ik ben dan ook heel blij dat ik het CRABP-werk mag voortzetten binnen de onderzoeksgroep Embryonale Ontwikkeling, en stel mij veel voor van de directe samenwerking met deze mensen.

Hierbij wil ik ook mijn waardering uitspreken voor de plezierige contacten met mensen van andere afdelingen, zowel binnen de Erasmus Universiteit als daarbuiten. Vooral de samenwerking met het Hubrecht Laboratorium heb ik als zeer plezierig ervaren, en ik verheug me erop de samenwerking ook in de toekomst voort te zetten.

The Editorial Office of The Company of Biologists has been most helpful in providing me with the colour illustrations that go with Chapter IV and are included as inset. This kind gift is an example of the excellent service provided by the publishers of Development, for which I want to express my sincere gratitude.

Een aantal mensen was niet rechtstreeks betrokken bij het wetenschappelijk werk, maar heeft niettemin een belangrijke rol gespeeld bij de totstandkoming van het beschreven onderzoek. Daarmee doel ik op de mensen van het secretariaat - vooral Rita Boucke; de fotografen - vroeger Joop Fengler en Tar van Os, tegenwoordig Mirko Kuit, Tom de Vries-Lentsch, en Ruud Koppenol; de mensen van de keuken - Jopie Bolman, Elly Hoffman, Joke Bolman, en vroeger Mevrouw Godijn; en Piet Hartwijk van de werkplaats. Ik wil al deze mensen hiervoor hartelijk bedanken.

Wie tenslotte niet mag ontbreken in dit dankwoord, is Bobby. Zijn bijdrage in de vorm van mijn geestelijk en lichamelijk welzijn moet niet onderschat worden. Het leven bestaat uiteindelijk niet uit werk alleen.

Abbreviations

APL	acute promyelocytic leukemia
AER	apical ectodermal ridge
CAT	chloramphenicol acetyl transferase
CNS	central nervous system
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
DMSO	dimethyl sulfoxide
EC	embryonal carcinoma
pCaBP	placental Calcium binding protein
PCR	polymerase chain reaction
RA	retinoic acid
RACE	rapid amplification of cDNA ends
RAR	retinoic acid receptor
SSC	standard saline citrate
SDS	sodium dodecyl sulphate
ZPA	zone of polarising activity

