REGULATION OF THE GENE FOR CELLULAR RETINOIC ACID BINDING PROTEIN TYPE I. (CRABP-I)

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REGULATION OF THE GENE FOR CELLULAR RETINOIC ACID BINDING PROTEIN TYPE I.

(Regulatie van het gen voor Cellulair Retinoylzuur Bindend Eiwit type I)

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam,
op gezag van de Rector Magnificus
Prof. Dr P.W.C. Akkermans, M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 18 februari 1998 om 13:45 uur

door

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geboren te Maasland.

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Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica, Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam. De vakgroep maakt deel uit van het Medisch Genetisch Centrum Zuid-West Nederland. Het onderzoek werd financieel gesteund door de Nederlandse Hartstichting.

Gedrukt bij Offsetdrukkerij Ridderprint B.V., Ridderkerk

The larger the island of knowledge,
the longer the shoreline of wonder.

Old chinese proverb

Nature, it seems, is the popular name
for milliards and milliards and milliards
of particles playing their infinite games
of billiards and billiards and billiards

Piet Hein, 1966.

Voor mijn ouders.

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List of abbreviations

A	Adenine
AP-1	Activator protein 1
ATP	Adenosine triphosphate
bp	basepair
BSA	Bovine serum albumin
C	Cytosine
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
COUP	Chicken ovalbumin upstream promoter
cpm	counts per minute
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
DNAseI HSS	DNAse hypersensitive site
DORV	Double outlet right ventricle
DR	Direct repeat
DTT	Dithiothreitol
EDTA	Ethylenediaminotetra-acetic acid
EMSA	Electrophoretic mobility shift assay
G	Guanine
HRE	Hormone response element
Hsp	Heat shock protein
HSS	Hypersensitive site
kb	kilobase
kD	kiloDalton
LBD	Ligand binding domain
mRNA	Messenger Ribonucleic acid
NLS	Nuclear localisation signal
nt	Nucleotide
PAA	Polyacrylamide
PMSF	Phenylmethylenesulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
RA	All-trans retinoic acid
9-cis RA	9-cis retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Serum retinol binding protein
RNA	Ribonucleic acid
RXR	Retinoid X receptor
RXRE	Retinoid X response element
SDS	Sodium dodecylsulphate
T	Thymine
TF	Transcription factor
TR	Thyroid hormone receptor
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
TTR	Transthyretin
U	Uracil
UTR	Untranslated region
VDR	Vitamin D receptor
VSD	Ventricle septum defect

Amino acids

A	Ala	Alanine	N	Asn	Asparagine
C	Cys	Cysteine	P	Pro	Proline
D	Asp	Aspartic acid	Q	Gln	Glutamine
E	Glu	Glutamic acid	R	Arg	Arginine
F	Phe	Phenylalanine	S	Ser	Serine
G	Gly	Glycine	T	Thr	Threonine
H	His	Histidine	V	Val	Valine
I	Ile	Isoleucine	W	Trp	Tryptophan
K	Lys	Lysine	Y	Tyr	Tyrosine
L	Leu	Leucine			
M	Met	Methionine			

Scope of the thesis

Early this century the important biological role of vitamin A became recognised as a result of nutritional studies. Vitamin A (retinol) and its derivatives, collectively referred to as retinoids, exert a wide variety of effects on vertebrate development, cellular differentiation and homeostasis (Bollag, 1996). Retinoids, and most notably retinoic acids (RAs) have been of special interest to developmental biologists because of their teratogenic effects on fetal development. Either excess or deficiency of retinoids during pregnancy has been shown to lead to many birth defects (Wilson, 1953; Kochhar. 1967). Thus, normal development seems to require a careful balance of retinoids. How such structurally simple molecules as the retinoids can have such pleiotropic effects has been a long standing question. In recent years it has become clear that the mechanisms through which RA affects cellular differentiation and embryonic development involve complex interactions between the products of two distinct gene families. The first family consists of a number of nuclear receptors for retinoic acid, and belongs to the superfamily of steroid/thyroid hormone receptors. It comprises two groups of RA receptors, the RARs (a,ß,γ), which bind both all-trans RA and 9-cis RA, and the RXRs (a,ß,γ), which have 9-cis RA as their specific ligand. These receptors form heterodimeric complexes and act as ligand controlled transcription factors. The nuclear receptor heterodimers regulate gene transcription through binding to specific DNA sequences, termed RA response elements (RARE), found in the promoter regions of target genes.

The second family consists of serum and cytoplasmic retinoid binding proteins that belong to a superfamily of small proteins involved in the binding, transport, and/or metabolism of low molecular weight hydrophobic molecules. Two members of this family, the cellular retinoic acid binding proteins type I and II (CRABP-I and II) have been shown to interact specifically with RA. Both CRABPs are expressed independently, and they bind RA with high, but different affinities. Both CRABP genes show very specific expression patterns in adult tissues and during embryonic development. Their exact physiological role remains to be elucidated, but they are most likely involved in regulating the amount of RA that is available to the nuclear receptors.

When the project described in this thesis was initiated the cellular retinoic acid binding protein type I (CRABP-I) was thought to be an essential component of the RA signal transduction pathway. CRABP-I was first isolated in the mid 1970s by virtue of its high binding affinity for RA, and was at that time assumed to be the direct mediator of the effects of RA at the cellular level. However, the observations that in most cells CRABP is located only in the cytoplasm, that it lacks homology with any known transcription factor and is not expressed in all RA responsive. cell types, led to the conclusion that CRABP was not the direct mediator of RA action at the transcriptional level. In 1987 the first nuclear receptor for RA was isolated based on homology with the steroid hormone receptors, soon followed by the other members of the RAR family, and it became clear that these RARs were the factors that relayed the RA signal to the expression of target genes (Giguere, 1987; Petkovich, 1987). CRABP-I was believed to be critically involved in the regulation of the availability of retinoic acid for the nuclear receptors, but it was unclear how exactly it might do so. Thus even though much had become known about the protein concerning its binding properties and expression patterns at various stages of development, the function of CRABP-I remained unclear. The mouse CRABP-I gene had recently been cloned (Vaessen,1990; Stoner, 1990; Perez-Castro, 1990) and this allowed the detailed study of its expression pattern during embryonic development, as well as in adult tissues. Its highly spatio-temporally specific expression pattern in the developing

embryo appeared to indicate that CRABP-I serves a specific function during embryonic development. As expression of CRABP-I was found specifically in those structures that are most sensitive to excess of RA during development it was thought that CRABP-I would function as a cytoplasmic buffer, protecting these sensitive cells from excess retinoic acid.

The aim of the project described in this thesis was therefore to increase our knowledge of the regulation and the function of CRABP-I during embryonic development. During the course of the project a direct test of the function of the protein was undertaken by several groups, by generating mice in which the genes for CRABP-I alone, or in combination with CRABP-II, were functionally inactivated via gene targetting. Surprisingly, the absence of a functional CRABP-I protein in these mice did not give rise to any apparent abnormal phenotype, at least not under laboratory conditions. Yet, the evolutionary conservation of CRABP-I is extremely high, and it is expressed in a very specific pattern during embrogenesis, which suggests that the protein is somehow important for the organism. We then focussed our attention on the regulatory mechanisms that direct the specific expression pattern of the gene in the developing embryo. Initial experiments to identify the regulatory elements were conducted in cell lines expressing the gene at high levels. When these remained inconclusive we switched to the approach of generating transgenic mice to study the regulation of the gene. This would enable us to assess the expression of the gene directly in the complex situation of the whole embryo. To accompany the study of the regulatory mechanisms in transgenic mice we also performed an interspecies comparison of the promoter regions of the CRABP-I gene from the mouse, chicken and pufferfish, with the aim of identifying regions of high conservation between the various species that, presumably, would be involved in the transcriptional regulation of the gene. We anticipated that a better knowledge of the factors regulating CRABP-I expression would lead to a more complete understanding of the RA signal transduction pathway, and, furthermore, that it would enable us to study the function of CRABP-I in the embryo by directly modulating the level of CRABP-I in specific embryonic tissues.

CHAPTER I

General introduction

General introduction.

During embryonic development of vertebrate organisms a single fertilised egg is transformed into a complete organism through the complex interplay of developmental processes such as cell proliferation, migration, differentiation, apoptosis and morphogenesis. The developmental program responsible for the regulation of these processes is encoded in the DNA of the organism itself. In a multicellular organism all cells, with a few exceptions, contain a complete and identical copy of the genetic information, including this developmental program. The conversion of the abstract information encoded by the DNA into physiologically active proteins is called gene expression. Through the use of differential gene expression multicellular organisms can develop with a great diversity of different cell types, each fulfilling a specific function. Each of these cell types expresses a unique subset of the total number of genes. Many genes are always active in all cells of the organism. These genes are called house-keeping genes and most of them encode structural proteins and basic metabolic enzymes, important for survival of the cell. Other genes are expressed in only one, or a few specific cell types, and sometimes only during a particular stage of development. The regulation of these differentially expressed genes is tightly controlled by particular intra- and extracellular signals (Alberts, 1989), some of which may be epigenetic.

Development of multicellular organisms depends on complex regulatory networks in which specialised factors relay specific developmental signals to target genes in certain cell types. The development and morphogenesis of an organism depends on the correct interaction and integration of all these signals to execute the developmental program, and hence involves a strict regulation of the expression of all its developmentally important genes. The proteins involved in developmental gene regulation range from diffusable peptide hormones, their cell surface receptors, and intracellular signal transduction molecules to the so called transcription factors. These are DNA binding proteins that interact directly with specific DNA elements located in the regulatory regions of genes and activate their expression. In the past years a large number of proteins has been identified that are involved in the regulation of embryonic development at the molecular level. The proteins of the Retinoic Acid (RA) signal transduction pathway are prominent amongst the factors involved in controlling correct development.

Retinoids exert their influence on a large number of biological processes through their involvement in the transcriptional regulation of the expression of genes involved in those processes. Gene expression in general is primarily regulated by transcription factors, which bind to specific DNA elements (termed enhancers) in the promoter region of target genes and interact with other proteins to modulate the activity of the basal transcription factors and the RNA polymerase assembled at the proximal promoter of the gene (Maniatis, 1987; Ptashne 1988). A family of transcription factors has been identified that are specifically activated by the presence of retinoic acid (RA) in the nucleus of the cell. These factors are termed retinoic acid receptors (RARs) and they act as heterodimeric RA inducible transcription factors. They are part of the larger nuclear hormone receptor gene family, which also includes the steroid hormone, thyroid hormone and the vitamin D receptors (Evans, 1988; Mangelsdorf, 1995; Chambon, 1996). Many genes have been identified that are regulated by the activity of members of the retinoid receptor family in response to RA. Since retinoids are hydrophobic molecules and therefore poorly soluble in the aqueous environment of the cells and the circulation of the organism, a number of retinoid binding proteins have evolved, that take care of the proper storage, transport, and uptake of retinoids by the cells and regulate the availability of RA for the retinoid receptors in the nucleus. The Cellular Retinoic Acid Binding Protein type I (CRABP-I), belongs to the latter group of proteins, and is thought to be critically involved in regulating the amounts of RA

that are available for the nuclear receptors in the cells in which it is expressed. This introductory chapter gives an overview of the dramatic and widespread effects RA has on embryonic development, and of the current understanding of the molecular mechanisms behind these effects. The chapter starts with an introduction to vitamin A and retinoic acid, and the role of these molecules in normal embryogenesis and teratogenesis. In the middle part of the chapter the current knowledge on gene regulation by the nuclear retinoid receptors is reviewed. The end of the chapter gives a detailed introduction of the cellular retinoic acid binding protein type I (CRABP-I), and of the potential role of this protein in the RA signal transduction pathway.

I.1 Vitamin A and retinoids.

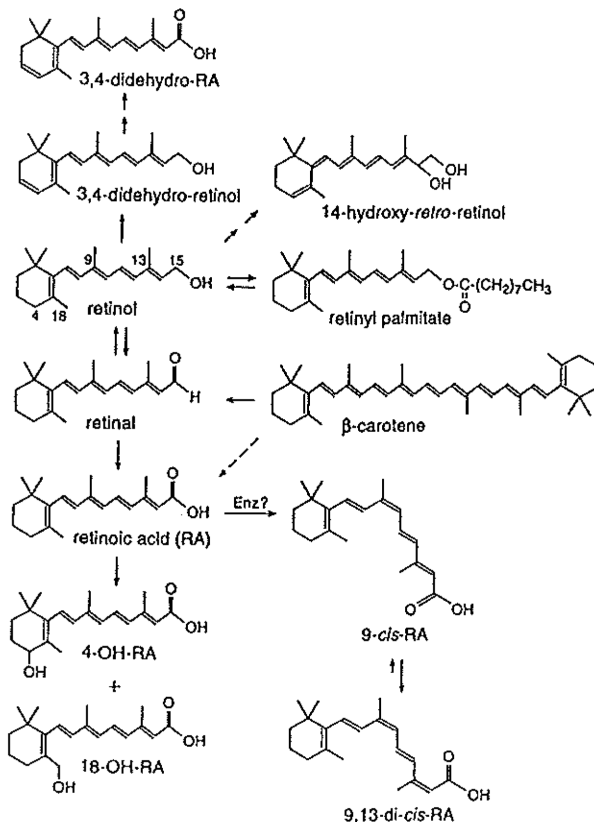

Fig. 1: Structures of naturally occurring retinoids.

Vitamin A plays a central role in many essential biological processes. Not only is it important for embryonic development in vertebrate organisms, but it is also involved postnatally in the regulation of proliferation, differentiation and apoptosis of many cell types, and in maintenance of the immune system. The vitamin A derivative retinal is of major importance as the chromophore required for vision (Wald, 1968). Vitamin A is the common name used to designate the molecule retinol. Nowadays however, the term vitamin A is also used to designate

any compound possessing the biological activity of retinol. The term Retinoids is used for all compounds that are structurally related to retinol. The Retinoids comprise both naturally occurring and synthetic compounds. Only a few members of this ever increasing group of compounds are in fact naturally occurring molecules (Figure 1). Most retinoids have been synthesised in the laboratory as potential candidates for anti-cancer therapies. This was spurred by the observations that vitamin A has a strong influence on cell proliferation and differentiation of certain cell types, making it a good candidate as a therapeutic agent for use in cancer therapy. The large number of side effects that treatment with high doses of vitamin A creates has led to an intensive search for more specific and powerful retinol analogues (Apfel, 1992; Lehmann, 1992, Armstrong, 1992, Lala, 1996).

Vitamin A has indirectly been known as a factor that can cure a deficiency disease for at least 3500 years. The ancient Egyptians recognised night blindness, which is caused by vitamin A deficiency, as a disease, and knew that it could be cured by eating ox liver, which, as we now know, is a rich source of vitamin A (Wolf, 1996). Vitamin A itself was first identified as an important factor for animal health in 1913. Rats fed on a diet of pure protein, pure milk sugar, minerals and olive oil failed to grow, but addition of butter fat to the diet restored their health. The fat-soluble factors of the butter could be extracted into ether, and when the extract was added to olive oil, the olive oil was now able to support growth on the original diet. This essential factor for growth and survival, ultimately shown to be retinol, was then named "fat soluble factor A", as opposed to other dietary factors called "water-soluble B" (McCollum, 1913). In 1930 it was shown that the yellow pigment, present in active extracts from plant sources, butter fat or egg yolk, and named carotene, is in fact a provitamin, which is converted in the body into the colourless active factor, vitamin A or retinol (Moore, 1930). Retinoic acid is the acid form of vitamin A, which arises from the oxidation of retinol via retinal. It was first obtained synthetically in 1941 in the form of the all-trans isomer, and its biological activity was tested in animal studies (Arens, 1941).

Transport and uptake of vitamin A.

A number of factors determine the amount of retinoids present in the cells of the organism: body intake, uptake, transport, storage, biosynthesis and degradation. Vitamin A is taken up by the body in the form of retinyl esters from animal sources, or in the form of carotenoids from plant sources. Beta-carotene is the most potent precursor for retinol, but it is still six-fold less effective than preformed retinol (Blomhoff, 1991). Retinyl esters and carotenes contained in the diet are converted into retinol or retinal in the intestine, and subsequently taken up by the enterocytes. The major conversion pathway of carotenoids in the intestine is by central cleavage of the carotenoid to produce 2 molecules of retinal, although some excentric cleavage, resulting in retinoic acid and retinal, may also occur (Wolf, 1996). In the enterocytes the retinol and retinal are bound by cellular retinol binding protein type II (CRBP-II). CRBP-II is a major intestinal protein and is present in high concentrations in the enterocytes. Binding to CRBP-II protects retinal from oxidation to RA, and allows it to be reduced to retinol by a microsomal retinal reductase (Kakkad, 1988). The retinol/CRBP-II complex is a substrate for the enzyme lecithin-retinol acyltransferase (LRAT), which converts retinol back to retinyl esters. These retinyl esters are then transported to the liver as a component of chylomicrons. Upon endocytosis of chylomicron remnants by the liver parenchymal cells the retinyl esters are converted back into retinol. From here the retinol is either transported to the target cells or, when sufficient retinol is already present in the circulation, stored in the liver stellate (fat storing) cells. For storage in these cells the retinol is once again converted into retinyl ester by

the enzyme LRAT. LRAT utilises lecithin as the acyl donor and requires retinol bound to cellular retinol binding protein type I (CRBP-I) as the substrate. Retinol can also be converted to retinyl ester by the enzyme AcetylCoA retinol acyltransferase (ARAT). ARAT can use only free retinol as substrate, and this conversion route is considered to be a very minor pathway (van Bennekum, 1993). When retinol is not stored in the liver, but transported to the target tissues immediately, it is loaded onto retinol binding protein (RBP), a 21 kD protein with a single binding site for retinol (Blaner, 1989). RBP is synthesised in the liver parenchymal cells. The RBP-retinol complex is secreted into the plasma for transportation. There the complex is bound by another protein, transthyretin (TTR), a 55 kD protein with high affinity sites for the thyroid hormones T_3 and T_4 (thyroxine). A 1:1 complex is formed between RBP and TTR, which prevents the excretion of the small RBP-retinol complex through glomerular filtration in the kidneys. At the target tissue the complex dissociates and retinol is taken up by the cells. Retinoids other than retinol, e.g. all-trans RA, 9-cis and 13-cis RA, 4-hydroxy-RA, 4-oxo-RA, have also been detected in plasma at low concentrations, complexed to albumin (Smith, 1973).

The mechanism by which retinoids enter the target cells is not yet clear. The existence of specific cell surface receptors that recognise the RBP-retinol complex has been suggested (Blaner, 1989; Blomhoff, 1992), and recently an RBP receptor has been reported in bovine retinal pigment epithelium (RPE) cells (Bavik, 1991). However, retinoids are small lipophilic molecules and could therefore also cross membranes spontaneously (Noy, 1992).

Upon entry into the cytoplasm of the target cell retinol is bound by cellular retinol binding protein type I (CRBP-I) (Ong, 1984). In view of the apparent importance of retinoic acid during development of the organism, and also postnatally in the control of the differentiation state or the entry into apoptosis of numerous cell types, it is reasonable to expect that the biosynthesis of RA and the rate of its turn-over are carefully regulated, to orchestrate its spatio-temporal concentrations. Retinol is converted into retinoic acid by two successive reactions, with retinal as an intermediate. A detailed knowledge of the enzymes involved in these conversions is only just beginning to emerge. The ability of retinoids to serve as substrates for many enzymes *in vitro* has made it difficult to establish which are the enzymes that are most likely to be physiologically relevant. In most cells the concentration of binding protein exceeds that of the retinoid, ensuring maximum binding of the retinoid to its binding protein. As a consequence, the CRBP-I/retinol complex is the most likely substrate for RA biosynthesis *in vivo*. CRBP-I has been shown to be involved in the conversion of retinol and retinal to retinoic acid (Posch, 1992; Ottonello, 1993). Both retinol and retinal, bound to CRBP-I, are substrates for specific cytosolic dehydrogenases. An NADP-dependent microsomal retinol dehydrogenase (RDH) catalyses the first reaction in this pathway. Retinal generated in microsomes from holo-CRBP by RDH is converted to RA in the cytosol by an NAD-dependent retinal dehydrogenase (RalDH) (Posch, 1991; Posch, 1992). CRBP may confer specificity on the biosynthesis of RA by restricting retinol access to specific enzymes. This would prevent opportunistic interaction of retinol with non-specific enzymes, and also protect retinol from non-enzymatic oxidation.

The conversion of retinol into RA results in a biologically active form of the retinoid, which exerts its effect by binding to the nuclear retinoic acid receptors (RARs and RXRs). The cellular retinoic acid binding proteins (CRABP-I and II) are involved in regulating the availability of RA for these nuclear receptors, but the mechanism by which they do so remains to be clarified. A number of possible roles for the CRABPs have been proposed. They may act (1) as modulators of RA metabolism, regulating intracellular RA concentrations, (2) simply as a cytoplasmic buffer for RA, (3) as a means to sequester RA in specific cells, or (4) as a nuclear translocation system (Gustafson, 1996).

Metabolism of retinoic acid.

The first step in the metabolism of retinoic acid is the oxidation of the beta-ionone ring to form hydroxy RAs. Depending on the tissue, hydroxylation is initiated at one of two different beta-ionone ring sites: C4 and C18, to produce 4-hydroxy-RA or 18-hydroxy-RA. 4-hydroxy-RA undergoes further conversion into 4-oxo-RA (Fiorella and Napoli 1994). It remains to be established whether these 4-oxo-RA and 4-OH-RA meta-bolites are simply intermediates in the

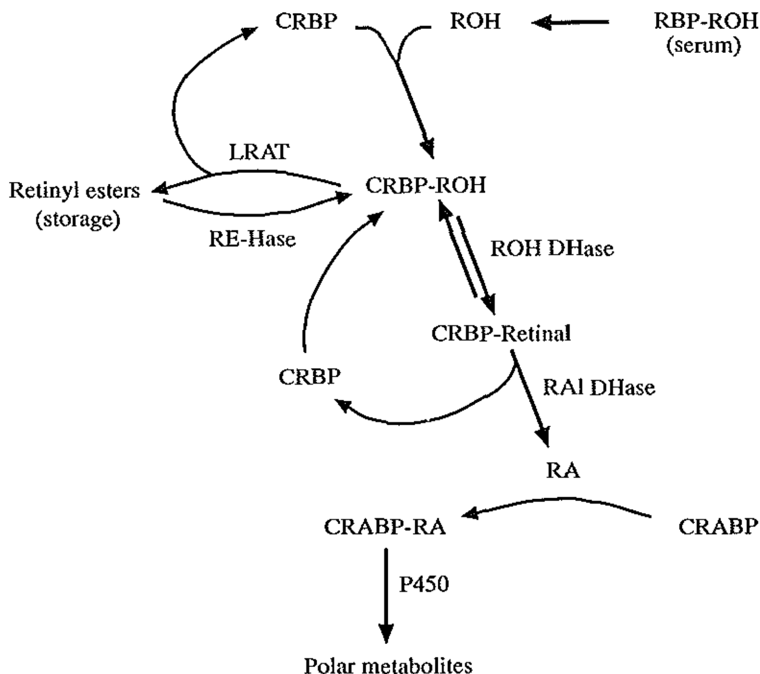

Fig. 2: Schematic representation of the metabolic and catabolic pathways of retinoic acid. ROH = retinol, RA = retinoic acid, CRBP = cellular retinol binding protein, CRABP = cellular retinoic acid binding protein, RBP = serum retinol binding protein, ROH Dhase = retinol dehydrogenase, Ral Dhase = retinal dehydrogenase, LRAT = lecithin/retinol acetyltransferase, RE-Hase = retinyl ester hydrolase. (Adapted from a model by Napoli, 1993).

RA catabolic pathway, or whether they have specific activities different from all-trans RA and 9-cis RA. 4-oxo-RA binds to RARß with an affinity that is comparable to all-trans RA, but it binds poorly to RARγ (Pijnappel, 1993; Reddy, 1992). 4-oxo RA also binds to the CRABPs, with an affinity that is only slightly lower than that of all-trans RA (Duell, 1992; Fiorella, 1993). It has been shown that all-trans 4-oxo RA is a highly active modulator of positional specification in early *Xenopus* embryos (Pijnappel, 1993). Furthermore, in the induction of proliferation of growth arrested A spermatogonia in vitamin A deficient mice 4-oxo RA is more potent than RA (Gaemers, 1996).

The catabolism of RA to polar metabolites is believed to be a cytochrome P450

dependent process. This idea follows from the observation that the general cytochrome P450 inhibitors ketoconazole and liarozole effectively inhibit the production of polar RA metabolites (Williams, 1987; Van Wauwe, 1988, 1990, 1992). In a number of tissues and cell lines RA metabolism can be induced by pre-treatment of the cells with RA, which suggests that at least some of the enzymes involved in RA metabolism are induced by RA itself (Roberts, 1979; Duell, 1992; Wouters, 1992; Takatsuka, 1996).

One of the hypotheses about the function of CRABP-I is that it is involved in RA metabolism. This hypothesis stems from the observation that CRABP-bound RA has a lower K_m (approx. 2 nM) for microsomal RA metabolism than unbound RA (approx. 50 nM) (Fiorella, 1991). Furthermore, overexpression of CRABP-I in F9 cells resulted in a shorter elimination half-time of RA, consistent with holo-CRABP being a more efficient substrate for RA metabolism than free RA (Boylan, 1992). However, the 40 minute elimination half-time of RA, incubated in the presence of microsomes, was not altered by the addition of apo-CRABP-I (Fiorella, 1994).

Hydroxy-RAs also bind to CRABP, but are metabolised very slowly in the CRABP-bound form. In their free forms both 18- and 4-hydroxy-RA are rapidly metabolised, with elimination half-times of approximately 35-40 minutes (Fiorella, 1991, 1994). Similarly, the elimination half-time of 4-oxo-RA is 9 minutes, whereas the half-time rises to over 10 hours when it is bound to CRABP. These observations suggest a role for CRABP in the differential presentation of selected retinoids for metabolism by the P450 system. Figure 2 shows a schematic representation of the various metabolic pathways of retinol and retinoic acid.

Retinoic acid and teratogenesis.

Vitamin A plays an important role in the embryonic development of vertebrate animals. It is critically required for many of the processes that take place in the embryo during its development. Vitamins, by definition, are molecules that are essential for the organism, but cannot be synthesised *de novo* by the animal itself. They are therefore essential components of the diet. Embryos depend on the availability of vitamin A in their immediate environment. In mammals retinol is supplied to the embryo by the mother, first via the yolk sac and later via the placenta. Both deficiency and excess of vitamin A during pregnancy lead to a large number of congenital malformations. The importance of vitamin A for embryonic development was first implied by experiments with vitamin A deficient pigs (Hale, 1938). Foetuses of the vitamin A deficient animals showed a wide spectrum of malformations. The occurrence of these malformations could be prevented when retinol was administered to the pregnant mother. Furthermore, all the effects of vitamin A deficiency, with the exception of the effects on vision, could also be prevented by administration of retinoic acid, which showed that RA is probably the bio-active metabolite of vitamin A.

The teratogenic effects of vitamin A were first shown more than 40 years ago in rats treated with an excess of the vitamin (Cohlan, 1953). Numerous studies since then have shown that vitamin A excess during pregnancy leads to a wide variety of malformations, which have been well characterised. Comparison of the morphological effects of vitamin A deficiency (Wilson, 1953; Takahashi, 1975) and excess (Shenefelt, 1972; Morriss-Kay, 1991; Kessel, 1991) in rodents shows that the patterns of malformations induced by deficiency or excess are slightly different. While both deficiency and excess affect many of the same tissues, the frequency with which particular malformations occur is different. Following vitamin A deficiency, the most frequently observed abnormalities are in the development of the eye,

including anophtalmia (absence of the eye), the genito-urinary tract, the diaphragm, the heart (e.g. interventricular septum defect) and the lung. Following exogenous vitamin A treatment a characteristic pattern involving craniofacial, cardiac, thymic and central nervous system structures is often seen.

All-trans Retinoic acid is a much more potent teratogen (20-40 times greater) than retinol in many species. The spectrum of malformations found after RA treatment is similar to that observed after vitamin A treatment. Several observations argue that RA is the primary bio-active molecule responsible for the vitamin A induced teratogenesis: (a) Retinyl esters and retinol are readily oxidised to all-trans RA in the body; (b) all-trans RA is the most potent natural teratogenic retinoid; (c) all-trans RA has been demonstrated to be a ligand for the retinoic acid receptors.

A number of factors influence the nature and severity of anomalies found after treatment with exogenous vitamin A compounds. Firstly, the anomalies observed are dependent on the developmental stage at the time of retinoid administration. The period of organogenesis is the most sensitive period for exposure to teratogenic levels of retinoids (Soprano, 1986). Treatment of pregnant mice before implantation of the embryo (i.e. 4.5 days p.c.) or after embryonic day 14 (14 days p.c.) results in few, if any, adverse effects. However, treatment of pregnant mice between 7 and 10 days p.c. results in anomalies of the central nervous system, sensory organs and the cardiovascular system, in addition to a large number of resorbed embryos. Treatment between 10 days and 13 days p.c. results in malformations of the limb, the craniofacial area and the genito-urinary tract. Secondly, the severity of the effects depends on which particular retinoid the embryo has been exposed to. As mentioned above some retinoids are more potent teratogens than others. In addition, there appears to be a certain degree of species specificity in the potency of some retinoids. However, for all retinoids the teratogenic response is dependent on the dose. Both the frequency and the severity of the embryonic effects increase with an increase of the dose of the retinoid.

Retinoids as morphogens in normal development

During early embryogenesis axes are established within the embryo. The anterior/posterior (A/P) axis and the dorsal/ventral (D/V) axis are first set up in the central body of the embryo, distinguishing head from tail and back from front. Later in development the limb buds start to grow, and there a proximo/distal (P/D) axis must be formed in addition to the A/P and D/V axes. Exogenously added retinoids cause malformation of structures that arise along the A/P axis of the central body, as well as abnormal development of the limb bud. Numerous studies implicate retinoids as morphogens, involved in establishing positional information along the A/P axis in the developing central nervous system (CNS) and the vertebral column. At a later stage in development retinoids have been implicated in A/P axis specification of the limb bud, where a specific pattern of digits along this axis is formed (see separate paragraph). The influence of retinoids on pattern formation in the CNS has been primarily investigated in the mouse and in the African clawed frog, *Xenopus laevis*. Axial patterning in vertebrates is set up during gastrulation. The site of initial invagination during gastrulation in *Xenopus* is called the organiser, and this site becomes the posterior end of the embryo. The corresponding structure serves the same function in mammals, where it is called the node, and in birds, where it is called Hensen's node. These structures have all been shown to be sources of endogenous active retinoids (Rossant, 1991; Hogan, 1992; Chen, 1992a/b; Balkan, 1992). Exogenous retinoids not only interfere with gastrulation but also influence subsequent processes in A/P determination. Ectopic addition of RA to *Xenopus* embryos at neurula stages causes posteriorisation of anterior

structures (Durston, 1989; Sive, 1990; Ruiz-i-Altaba, 1991; Papalopulu, 1991; Blumberg, 1997). The RA metabolite 4-oxo RA has been shown to be equally potent in causing these A/P defects (Pijnappel, 1993). Interestingly, overexpression of its only known CRABP, xCRABP, in *Xenopus* causes anterior/posterior defects similar to those observed after RA treatment (Dekker, 1994).

In mammals the most obvious effects of RA on A/P axis formation are seen in formation of the hindbrain neuroepithelium and its associated neural crest. Neural crest cells derived from the hindbrain region migrate to many structures in the face, throat area and heart, and contribute to correct formation of these structures. The hindbrain is divided into eight morphologically distinct regions called the rhombomeres. These rhombomeres each have their own unique identity, which is reflected in the expression of specific marker genes, e.g. Hox genes, Krox20, CRABP-I. At high doses RA abolishes this rhombomere segmentation. At lower doses of RA the rhombomere identity is altered in such a way that anterior rhombomeres acquire the identity of more posterior rhombomeres (Papalopulu, 1991; Marshall, 1992; Morriss-Kay, 1991). It is obvious that a highly controlled level of retinoids is involved in the regulation of the development of the hindbrain. The influence of retinoids on the development of this region may be mediated through their involvement in the regulation of expression of a specific group of homeotic genes, called the Hox genes.

Homeotic genes encode a class of transcriptional regulators that were first identified in the fruitfly, *Drosophila melanogaster*, as genes that specified the identities of the different regions or compartments of the body (Lewis, 1978, Lewis, 1992). Mutations in these homeotic genes have the effect of transforming one body part into the likeness of another. Homeobox genes encode transcription factors that have a special DNA binding domain, called the homeobox. In *Drosophila* a particular set of these homeobox genes are organised in two genomic clusters, the Antennapedia cluster (ANT-C) and the Bithorax cluster (BX-C), together referred to as the homeotic complex (HOM-C). Vertebrates have conserved these genes throughout evolution. Four sets of linked homeobox genes, called the Hox genes, are organised into four different chromosomal complexes, containing genes related to the homeobox genes of both the ANT-C and BX-C clusters (Figure 3). Their structural and organisational similarities suggest that the Hox gene complexes evolved by duplication and divergence from a common ancestral gene cluster (Kappen, 1989; Ruddle, 1994). As with the *Drosophila* HOM-C cluster genes the vertebrate Hox complexes tend to be expressed in a colinear fashion, with the genes located at the 3' end of the cluster being expressed in more anterior compartments of the embryo, and the more 5' located genes limited to more posterior compartments (Krumlauf, 1994; Machonochie, 1996). In vertebrates an additional type of colinearity exists based on the time of appearance of expression during embryogenesis. More 3' located genes start to be expressed earlier than those that are located more 5' in the complex. Thus the physical order of the genes in the complex is reflected both in their anterior boundary of expression and in the sequence of their temporal activation (Figure 3). The partially overlapping expression domains of the Hox genes provide a mechanism for the specification of positional information in the embryo, which is usually referred to as the "Hox code" (Lewis, 1978). Retinoids appear to be endogenous signalling molecules that are involved in the sequential anterior/posterior activation of Hox genes in the neural tube. Three lines of evidence support this notion. Retinoic acid response elements (RAREs) have been identified in several of the Hox genes (Langston, 1992; Popperl, 1993; Moroni, 1993; Marshall, 1994; Langston, 1997). The presence of these RAREs has been shown to be required for correct expression of the genes in the case of the HoxB1 and HoxA1 genes (Marshall, 1994; Ogura, 1995a/b; Frasch, 1995; Dupé, 1997). The HoxA1 RARE

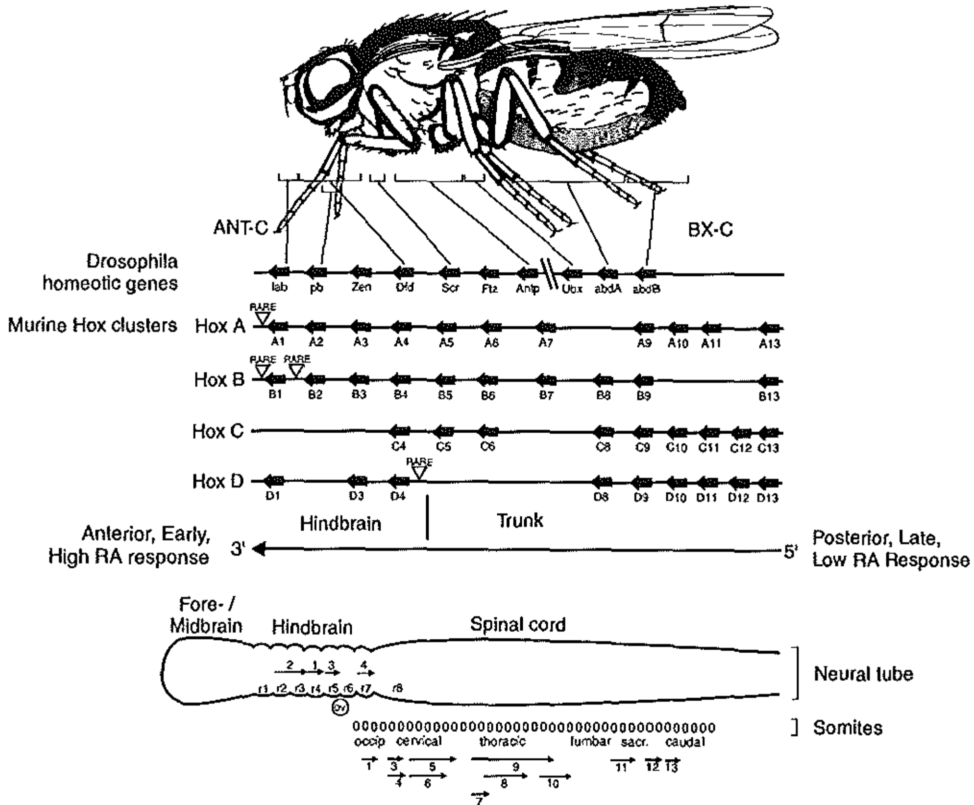

Fig. 3: The Hox gene clusters in mice and *Drosophila*. Hox genes are expressed collinearly along the anterior/posterior body axis. (A) The murine Hox gene clusters (HoxA-D) are indicated beneath their homologous *Drosophila* counterparts. For the murine clusters each column represents a paralog group (e.g. HoxA3, HoxB3 and HoxD3 form a paralog group). The positions of known RA response elements (RARE) in the clusters is indicated. (B) The mouse central nervous system and somites are diagrammed. The arrows indicate the anterior boundaries of Hox paralogous genes in the hindbrain and in the somites. Note that the arrows indicate only the anterior boundaries and that expression domains extend much further posteriorly. (Adapted from Means, 1995).

is necessary for the response to RA both in cell culture and in mice (Langston, 1992; Frasch 1995), and deletion of this RARE by gene targeting in mice affects both HoxA1 and A2 expression, resulting in hindbrain malformations (Dupé, 1997). The HoxB1 gene contains multiple RA response elements, which regulate different aspects of its specific pattern of expression in the developing hindbrain (Marshall, 1994; Ogura, 1995a/b; Marshall, 1996). To date RAREs have been identified in the regulatory regions of the HoxA1, HoxB1 and HoxD4 genes, and these are all evolutionarily well conserved.

RA treatment of embryos alters Hox gene expression boundaries concomitant with homeotic transformations of the axial skeleton (Kessel, 1990/1991/1992; Morriss-Kay, 1991; Conlon, 1992; Marshall, 1992; Sundin, 1992; Kolm, 1995). RA treatment of embryos can induce a homeotic transformation in the hindbrain neuroanatomy and respecify rhombomeres 2 and 3 into a rhombomere 4 and 5 identity (Marshall, 1992). Furthermore, there is a correlation between the responsiveness to RA and the location of a given Hox gene in the complex. The

more 3' located genes in a Hox cluster are more sensitive to RA than the more 5' located genes (Simeone, 1990; Stornaiolu, 1990; Cho, 1990; Papalopulu, 1991; Dekker, 1993). Thus, in addition to the positional and temporal colinearity in the Hox clusters, a third type of collinearity exists with respect to the RA responsiveness of the genes.

Analogous to the situation in hindbrain and neural crest patterning, the influence of RA on A/P patterning of the vertebrate central body is also clearly seen in the patterning of the paraxial mesoderm, which is involved in the formation of the vertebrae. Vertebrae arise from pairs of somites, blocks of segmented paraxial mesoderm on either side of the neural tube. The occipital bones at the base of the skull are derived from the first four somites. The rest of the somites form the cervical, thoracic, lumbar, sacral and caudal vertebrae. Exogenous retinoids, at low doses, cause homeotic transformations of vertebral identity, reminiscent of the transformations seen in the hindbrain after RA treatment (Kessel, 1991). These transformations resemble those seen as a result of certain Hox gene mutations, suggesting that RA alters segment identity in the formation of the vertebrae altering Hox gene expression (Lufkin, 1992; Kessel, 1990; McLain, 1992; Pollock, 1992; Jegalian, 1992). In the somites, as in the neural tube, the Hox genes are expressed in a collinear fashion with the most 3' genes expressed earliest and most anteriorly, and the 5' genes being activated later and more posteriorly. The anterior expression boundaries of the Hox genes are however found more posterior in the somites than in the neural tube. After gastrular involution of the paraxial mesoderm is complete the Hox genes are downregulated (Kessel, 1991). RA exposure early in somite determination leads to posteriorising transformations, probably due to shifting Hox gene expression anteriorly early in gastrulation. RA exposure later in development, when Hox genes are being downregulated, causes anteriorising transformations in posterior regions, probably by extending the time of Hox gene expression (Kessel, 1991).

Retinoic acid in limb bud development.

The claim that RA could be a morphogen initially arose from the spectacular effects retinoic acid appeared to have on pattern formation of the chicken limb bud (Slack, 1979). Chicken limb buds are an easily accessible system to study pattern formation during development. They arise from small buds that protrude from the embryonic flank at the correct axial levels and consist of locally proliferating lateral plate mesoderm cells (Newman, 1988). Interactions with the overlying ectoderm induce the formation of a specialised structure called the apical ectodermal ridge (AER). Once the AER has formed, it is responsible for maintaining the continued outgrowth of the limb bud (Saunders, 1972), and for elaborating the pattern along the proximo/distal axis (Summerbell, 1973). Within the developing limb bud, a specialised region at the posterior margin of the bud, called the zone of polarising activity (ZPA), is believed to be responsible for the specification of pattern along the anterior/posterior axis. Transplantation of the ZPA to an anterior position in a host limb bud results in mirror image duplication of the limb along the A/P axis, reflected in the pattern of digits (Saunders, 1968). A model was proposed in which a morphogen released by the ZPA sets up a gradient across the limb bud, resulting in a high concentration near the ZPA and progressively lower concentration at greater distances from the ZPA (Tickle, 1975). A high concentration would specify posterior digits, and a lower concentrations would specify more anterior digits.

For many years RA was believed to be this putative morphogen, for a number of reasons: Retinoids were found to mimic the polarising effects of the ZPA. Beads soaked in RA (or 3,4-didehydro-RA) also caused mirror image digit duplications when they were grafted to the anterior margin of the limb bud. Furthermore, endogenous retinoids have been identified in

the limb buds of chicken and mouse embryos (Scott, 1994; Thaller, 1987). When a large number of anterior versus posterior halves of limb buds were compared, a two-fold higher level of RA was found in the posterior halves, suggestive of an RA gradient (Thaller, 1987). CRABP-I was reported to be present in the chicken limb bud in an opposing gradient, with the highest concentration of CRABP-I anteriorly (Maden, 1988; Maden, 1994; Perez-Castro, 1989). CRABP-I would thus help to steepen the gradient of free RA across the A/P axis of the limb bud. However, in several other studies in mouse limb buds the putative gradient along the A/P axis could not be detected (Dolle, 1989; Miyagawa-Tomita, 1992; Ruberte, 1992), in agreement with our own observations (This thesis). The discrepancy may reflect a difference between species, since the gradient of CRABP expression was found in chicken limbs, whereas no A/P gradient was found in the mouse.

More recent studies on RA in limb buds indicated that rather than acting as a morphogen, RA may act to induce a new organising centre when implanted as a RA soaked bead in a chick limb bud. This newly induced ZPA would then emit the true morphogen. It was shown that the tissue adjacent to the implanted RA bead could, when transplanted to a new host, act as a ZPA (Wanek, 1991). Whereas an RA soaked bead induces RARß expression in the host limb bud, the RA induced ZPA does not induce RARß in the new host, arguing against the view that the ZPA would emit RA (Noji, 1991).

After the discovery of Sonic Hedgehog (SHH), a secreted protein, this molecule has become a more likely candidate to be the endogenous morphogen in the limb bud. SHH is expressed in the ZPA and it is sufficient to convey polarising activity when misexpressed in the limb bud (Riddle, 1993). RA can induce expression of SHH, so grafting an RA soaked bead in the anterior margin of the limb bud may create a ZPA because of the induction of SHH expression in the cells around the bead. It remains a controversial matter whether RA is acting before SHH in the endogenous situation in the limb, or whether this effect of RA is an artefact (Riddle, 1993; Chen, 1996). The latter view is held in a currently favoured model, where RA is only involved in limb bud initiation, possibly via activation of FGFs and certain Hox genes in the paraxial mesoderm, specifically HoxB8 and HoxC8 (Charite,1994; Tabin, 1995). Grafting RA soaked beads in the limb bud activates this program of gene expression in the wrong place, leading to aberrant activation of Sonic Hedgehog expression.

With respect to RA teratogenesis there appears to be a difference between the forelimbs and the hindlimbs. Vitamin A deficiency leads to limb malformations only in the forelimb (Morriss-Kay, 1996), and both the RARα/γ double null mutant and the CRABP-II null mutant have a limb defect exclusively on the forelimb (Lohnes, 1995; Mendelsohn, 1994). On the other hand, exposure to RA excess during early, pre-gastrulation stages of development leads to formation of a duplicated hindlimb, caudal and ventral to the normal limb, but there is no effect on the forelimbs (Rutledge, 1994). RXRα deficient mice have normal limbs, but are resistant to the adverse effects of RA on limb formation, showing that RXRα is required for RA signalling in limb teratogenesis (Sucov, 1995).

Retinoids and heart development

Heart development is amongst the processes that are influenced by retinoic acid. The development of the heart during embryogenesis involves both mesodermal and neural crest derived cell types. Mesodermal cells form a straight heart tube which undergoes rightward looping (leftward in birds), followed by formation of the atrial and ventricular chambers, while neural crest cells populate the developing aortic arches. Both vitamin A deficiency (VAD) and excess give rise to congenital heart malformations in many species (Wilson, 1953; Lammer,

1985; Hart, 1990; Dersch, 1993; Twal, 1995; Maden, 1996). A continuous spectrum of cardiac malformations, including Double Outlet Right Ventricle (DORV) and Ventricular Septum Defect (VSD), is found after administration of RA to developing chicken embryos (Hart, 1990; Broekhuizen, 1992, Bouman, 1995). These malformations have been proposed to be caused by a disturbance of the looping process of the heart tube and by malalignment of the outflow tract septum (Bouman, 1995; Broekhuizen, 1995). Neural crest cells that migrate from the cardiac neural crest region in the hindbrain contribute to the formation of the outflow tract region of the heart and influence septation of the outflow tract (Kirby, 1990/1995).

The use of excess RA treatment to induce a specific and reproducible spectrum of heart malformations is well established, but it remains questionable whether the results from RA excess studies are valid indications of the role of RA in normal heart development. Studies on a number of organisms fed on a vitamin A deficient diet (VAD) have shown that VAD also causes heart abnormalities (Wilson, 1949; Hale, 1928; Dickman, 1996). Heart defects seen after VAD include dysmorphic development of the cardiac mesoderm and defects in those structures of the heart that receive contributions from the cardiac neural crest. These include the truncus arteriosus, which is the common precursor of the pulmonary artery and the aorta, and the aortic arch system.

The role of the retinoid receptors in murine development has been explored by systematic gene targeting of the members of the retinoid receptor gene family. While mice with knockouts of one of the various RAR genes display no obvious cardiovascular defects, heart defects are found in mice, which lack certain combinations of these RA receptor genes. The RARα/RARγ and RARα1/RARß double knock-out mice show malformations of the aortic arches and the outflow tract of the heart. The abnormalities in these compound RAR null mutants are similar to the defects found after ablation of the cardiac neural crest in the chick (Kirby, 1995). When the RXRα gene is knocked out embryos exhibit a defective development of the ventricular myocardium, resulting in embryonic lethality by 14.5 days p.c. (Dyson, 1995; Kastner, 1994; Luo, 1995; Mendelsohn, 1994; Sucov, 1994). In addition to the severe cardiac muscle defects the RXRα deficient embryos also display abnormalities of the endocardial cushion tissue of both the atrioventricular region and the conotruncus (Gruber, 1996). The RXRα deficient mice thus exhibit heart abnormalities in non neural crest derived structures. Crosses resulting in RXRα/RARα or RXRα/RARγ double null mutants display the same thinner myocardium observed in the RXRα knock-out embryos, but also have a persistent truncus arteriosus (PTA), i.e. the absence of the aortico-pulmonary septum, sometimes in combination with various aortic arch anomalies (Kastner, 1994). It thus appears that RARs affect heart development via the neural crest, while RXRa is critically required for non neural crest related processes. Taken together these findings clearly show the importance of retinoid signalling pathway(s) in the formation of the heart.

I.2 The retinoid receptors.

Retinoic acid is a structurally simple molecule, and yet it exerts a wide variety of effects on vertebrate development, cellular differentiation and homeostasis. For many years the question has been how such a simple molecule as RA is able to elicit such a diversity of complex responses. Initially it was thought that the CRABPs were the primary mediators of the effects of RA. However, the picture of the operational mechanisms by which RA exerts its effects became clearer with the isolation of a nuclear receptor for retinoic acid (Giguere, 1987; Petkovich, 1987). This first RA receptor (RAR), now termed RARα, was isolated using a probe based on

the highly conserved DNA binding domain of other members of the steroid hormone receptor superfamily. Following the isolation of RARα two further RARs have been isolated, termed RARß (Brand, 1988) and RARγ (Zelent, 1989). Later, a second group of retinoid binding receptors was identified, named the retinoid X receptors (RXRs). As with the RARs, three different RXR genes have been identified (Hamada, 1989; Mangelsdorf, 1990; Mangelsdorf, 1992; Yu, 1991; Leid, 1992). Their name, retinoid X receptors, reflects the fact that at the time of their discovery the endogenous ligand for these receptors was not known. It was suspected to be a retinoid since RXRs responded to high concentrations of all-trans RA by transactivating a reporter gene in transient transfection assays (Mangelsdorf, 1990). Later the ligand for the RXRs was identified as 9-cis RA, a stereoisomer of RA (Levin, 1992; Heyman, 1992). Interestingly, whereas all-trans RA activates only the RARs efficiently, 9-cis RA is able to activate both RARs and RXRs. Based on sequence and structural homology the retinoid receptors have been grouped into the superfamily of nuclear ligand-activated transcriptional regulators. This large gene family includes the receptors for all steroid hormones (glucocorticoids (GR), mineralocorticoids (MR), progesterone (PR), androgens (AR), estrogens (ER)), as well as the thyroid hormone receptors (TR), vitamin D receptor (VDR), the peroxisome proliferation activating receptors (PPAR) and a large number of "orphan receptors", receptors for which the ligand, if any, has not been identified (Laudet, 1992; Mangelsdorf, 1996). Based on certain characteristics the hormone receptor superfamily can be divided into two broad subgroups, the type I and type II receptors. Receptors of the type I subgroup, which comprises all the steroid hormone receptors, contain a GSCKV DNA binding domain (DBD) motif, and bind to a AGA/GACA consensus half-site recognition sequence. These receptors are found both in the cytoplasm and in the nucleus, and are complexed to heat-shock proteins in the absence of hormone. The heat shock proteins are dissociated upon hormone binding, resulting in the formation of receptor homodimers on palindromically arranged hormone response elements. The type II receptors subgroup consists of the RARs, RXRs, VDR, TRs and PPARs. They bind DNA preferentially as heterodimers complexed with RXR and may do so even in the absence of ligand.

Functional organisation of the retinoic acid receptors

The members of the nuclear hormone receptor superfamily share a common architecture, which has been divided into six prominent structural domains, termed A to F. The RARs and RXRs share this modular structure, although the RXRs lack the F domain. The various domains contain regions that are important for DNA binding, ligand binding, transactivation/repression, nuclear localization and dimerisation (Figure 4). The C,D and E domains show in general the highest degree of conservation. The A/B and F domains are less conserved between members of the same class of receptors, but are conserved between the same receptors in different species. The amino-terminal A regions of all retinoid receptors are completely unrelated, and isoforms, differing in this region have been found for all RARs and RXRs (Giguere, 1990; Kastner, 1990; Leroy, 1991; Zelent, 1991; Nagpal, 1992; Liu, 1993; Nagata, 1994). These isoforms arise both through alternative splicing and from the differential usage of two promoters. There are two major isoforms for RARα (RARα1,2) and RARγ (RARγ1,2), and four major forms for RARß (RARβ1-4). Similarly, two major isoforms exist for each of the RXRs (Fleischhauer, 1992; Leid, 1992; Liu, 1993; Nagata, 1994). The exact role of the A/B domain is not entirely clear yet, but an independent transcriptional activation function, called AF-1, has been mapped to this region (Folkers, 1993; Nagpal, 1992/1993). Distinct spatio-temporal expression patterns for each RAR or RXR type and isoform, both in the embryo and in the adult, have been found.

Together with their strong evolutionary conservation this suggests a specific function for each isoform.

The 66 amino acid C region contains the DNA binding domain (DBD). This region is highly conserved among all retinoid receptors and is responsible for the specific recognition of the response elements found in the promoters of target genes. It comprises two zinc finger motifs (CI and CII), in which zinc is coordinated by four cysteine residues (Umesomo, 1988; Naar, 1991). Three amino acids in the CI finger, called the P-box, are critical for recognition of the half-site sequence of a response element, while 5 amino acids in the CII finger are thought to be important for recognition of the spacing between the half sites (Mader, 1989; Umesomo, 1989).

Region D is considered the hinge region between the DNA binding domain (C) and the ligand binding domain (E). This region includes the nuclear localisation signal (NLS). In contrast to the steroid hormone receptors, which are primarily found in the cytoplasm in the absence of ligand, the RARs/RXRs are constitutively present in the nucleus (Dalman, 1991). It should be noted that the RARs/RXRs (as well as the VDR, PPARs and TRs) do not interact with heat-shock protein complexes, unlike the steroid hormone receptors (Dalman, 1991). The D region of the RARs and RXRs is generally well conserved between different species (Leid, 1992).

The 220 amino acid E region is functionally complex, since it contains the ligand binding domain (LBD), the ligand-dependent transactivation function AF-2, and the dimerisation domain. It is also highly conserved between the three RAR genes and the three RXR genes. The dimerisation domain in the E region is the major protein-protein interface for dimerisation of RARs and RXRs in solution (Glass, 1990; Marks, 1992). Once contact has been made through this dimerisation domain the contact may be further stabilized through interprotein contacts within the C region and by interaction with DNA (Glass, 1994). Dimerisation of receptor monomers is a prerequisite for efficient DNA binding and transactivation. Nine highly conserved heptad repeats have been identified in the E region, and these have been shown to be required for efficient dimerisation between receptors (Forman, 1989; Forman, 1990a/b; Au-Fliegner, 1993). These repeats contain hydrophobic residues at positions 1 and 8, and hydrophobic or charged amino acids with a hydrophobic side chain at position 5 (Forman, 1990). This succesion of hydrophobic residues may fold into a leucine-zipper like structure or into a helix-turn-zipper motif (Maksymowych, 1993), resembling dimerisation interfaces found in other transcription factors. The RXRs heterodimerise with a number of other nuclear hormone receptors but in the presence of 9-cis RA they can also form homodimers (Zhang, 1992b; Zhang, 1993). It has been shown that the RXRs use distinct but possibly overlapping regions in the E region for hetero- and homodimerisation (Zhang, 1994).

The function of the F region, if any, is unknown. The F regions of the RAR genes are completely unrelated, and the RXRs do not contain an F region.

Signal transduction by the retinoid receptors

The retinoid receptors regulate gene transcription by binding as dimers to specific DNA sequences called RA response elements (RAREs) found in the regulatory regions of responsive genes (see separate section on the response elements). Upon binding of their cognate ligand they transactivate transcription of the target gene. The RXRs play a very central role in nuclear receptor signalling mechanisms. They have been identified as the heterodimeric partner, not only for the RARs, but also for TRs, VDRs, PPARs and a number of orphan receptors (Yu, 1991; Leid, 1992 ; Zhang, 1992 ; Kliewer, 1992 ; Marks, 1992; Bugge, 1992).

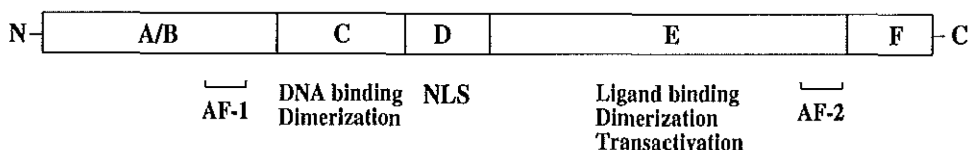

Fig. 4: Schematic representation of the functional domains of the retinoic acid receptors (RARs and RXRs). The variable A domain distinguishes receptor isoforms from each other, contains putative phosphorylation sites, and together with the B domain contains a ligand-independent activation function (AF-1). The two zinc-fingers involved in DNA binding reside in the C domain, as well as a weak dimerisation region. A nuclear localisation signal (NLS) is present in the D domain. The E domain contains multiple overlapping activities involved in ligand binding, dimerisation (via nine heptad repeats) and the ligand dependent transactivation function (AF-2). The F domain is absent from the RXRs and no function has been ascribed to it for the RARs.

RAR and TR homodimers bind very poorly to RAREs, and require heterodimerisation with RXR for efficient binding to their response elements (Zhang, 1991). In addition to their role as promiscuous heterodimeric partners the RXRs can also function as homodimers in the presence of their ligand 9-cis RA. RXR homodimers were found to bind and activate specific response elements of the DR-1 type, as found in the ApoAI and CRBP-II genes (Mangelsdorf, 1991; Zhang, 1992b).

Recently, a number of factors has been identified that interact with the receptors. Some of these proteins act as coactivators, mediating the activity of the activation functions AF-1 and AF-2 on the general transcription machinery, while others function as corepressors (Glass, 1997; Chambon, 1996). Although there is some evidence that nuclear receptors can contact some of the basal factors of the transcription preinitiation complex directly (Schulman, 1995), it is believed that to achieve efficient hormone dependent transcription these additional coactivator proteins are required. RARß2 has been shown to interact with TFIID via an E1A-like factor (Berkenstam, 1992; Kruyt, 1993). Using the yeast two-hybrid system, a number of putative coactivators for the nuclear hormone receptors have now been isolated, such as Trip1/SUG1, TIF1 and TIF2 (Bauer, 1996; Onate, 1995).

In contrast to most other nuclear receptors, unliganded RARs can strongly transrepress basal promoter activity of target genes in transfected cells. Two factors that bind efficiently to unliganded RARs, and are released upon ligand binding have been isolated via yeast two hybrid screenings. They are termed Nuclear Corepressor (N-CoR), and Silencing Mediator for RAR and TR (SMRT) (Kurokawa, 1995; Chen, 1995; DiRenzo, 1997). N-CoR may act as a polarity specific silencing mediator as it is released, in the presence of ligand, from an RXR/RAR heterodimer bound to a DR-5 response element, where RAR occupies the 3' half-site, but not from an RAR/RXR heterodimer bound to a DR-1 element, where the RAR occupies the 5' half-site (Kurokawa, 1995). The current view, at least in the case of an RAR/RXR heterodimer on a DR-5 response element, is that when both receptors are unliganded a corepressor masks the transcriptional activation function of the heterodimer (Baniahmad, 1995). Binding of ligand to the RAR then induces a conformational change which releases the corepressor and allows binding of a coactivator, resulting in transcriptional activation. Figure 7 integrates the current knowledge on the mechanism of gene activation by the retinoid receptors.

Cross modulation

COUPα and COUPß are orphan receptors related to the chicken ovalbumin upstream promoter transcription factor COUP-TF, which bind strongly to some, but not all RAREs. The response elements that can be bound by COUPs were efficiently inhibited in transient transfection assays (Tran, 1992; Cooney, 1992; Kliewer, 1992). These data suggest that the COUP receptors are repressors that can act by binding to a specific set of RA response elements, thus blocking the access of RAR/RXR heterodimers. In this way they can restrict RA signalling to a subset of RA responsive genes. The COUP-TF binding site in the ovalbumin promoter can also function as a RARE (Tran, 1992).

An additional level of complexity in the retinoid signalling pathway was introduced by the discovery of cross-modulation between the RA and the AP-1 signal transduction pathways (Nicholson, 1990; Lafyatis, 1990). While the nuclear receptors mainly regulate cell differentiation programs in response to their ligands, AP-1, when stimulated by extracellular signals, predominantly induces genes that are involved in cell proliferation. Activator protein 1 (AP-1) consists of two dimerised members of the c-Fos and c-Jun family of proto-oncogenes and binds to a DNA sequence motif termed AP-1 binding site or TPA-responsive element (TRE). By binding to TREs AP-1 mediates signals from growth factors, inflammatory peptides, oncogenes and tumor promoters. The TRE is not recognised by the retinoid receptors. The RARs and RXRs can, in the presence of their ligand, interact with AP-1 and inhibit its activity via a mechanism which appears not to involve DNA binding (Salbert, 1993; Schule, 1991; Yang, 1991). Via the same mechanism AP-1 can also inhibit RAR activity. Thus depending on the relative concentrations of the AP-1 components and the components of the RA pathway, a switch can be made between proliferation and differentiation (Shemshedini, 1991; Pfahl, 1993; Herrlich, 1994).

Response elements for the retinoid receptors

The retinoid receptors are (hetero-)dimeric transcription factors which act by binding to specific recognition sequences known as retinoic acid response elements (RAREs). These RAREs are found in the regulatory regions of target genes. The list of RAREs that have been identified still continues to grow, as the regulatory regions of more RA responsive genes are analysed (Figure 6). Genes containing RAREs in their regulatory regions include those for some of the isoforms of RARα, RARß, RARγ, CRBP-I, CRBP-II, CRABP-II, alcohol dehydrogenase 3 (ADH3), Laminin B1 and several Hox cluster genes. Most of these RAREs consist of an (imperfect) direct repeat (DR) of two half-sites with the 6 bp consensus recognition sequence 5'-PuGGTCA-3'. However, there are also RAREs, which consist of an everted repeat, an inverted repeat (palindromic) or a more complex configuration (see Figure 6). In almost all identified, natural RAREs at least one of the half-sites corresponds perfectly to the consensus sequence. The direct repeat RAREs are indicated, in general, by DR, followed by a number, in which DR stands for direct repeat, and the number indicates the length of the spacer region between the half-sites in nucleotides (e.g. DR5 means a direct repeat with 5 nucleotides spacing between the recognition half-sites).

Two factors appear to be important for DNA binding selectivity of the receptors to the RAREs. Firstly, not only the sequence of the half-sites, but also that of the flanking sequences influences the affinity of receptor binding (Mader, 1993; Nagpal, 1992; Predki, 1994). Secondly, the spacing between the half-sites appears to be important in specifying the particular type of receptor dimer that binds most effectively to the response element (Umesomo, 1991;

Mangelsdorf, 1994). The preferential response elements for the RAR/RXR heterodimers appear to be DR1, DR2 and DR5 elements, in order of increasing efficiency. Similarly, RXR/TR heterodimers bind preferentially to DR-4, RXR/VDR to DR-3 and RXR/PPAR to DR-1 elements (Umesomo, 1991; Mader, 1993; Mader, 1993; Glass, 1994). These observations have led to the formulation of the "1-2-3-4-5 rule". This rule-of-thumb reflects that response elements of a certain spacing have a preferred receptor heterodimer binding to them (Almasan, 1994) (Figure 5). The fact that RAR/RXR dimers can bind to DR1, DR2 and DR5 elements shows that a certain degree in flexibility is allowed in the response elements, at least in the case of the RARs.

The number of 9-cis RA response elements (RXRE) identified thusfar is more limited, and they all have a DR1 configuration. Thus DR1 elements are recognised by RAR/RXR heterodimers, but also by RXR homodimers or higher order RXR complexes (Mangelsdorf, 1991; Zhang, 1992). The promoter of rat CRBP-II contains a DR1 consisting of 4 directly repeated half-sites spaced by one cytosine residue. This complex binding site shows transactivation by RXRα. This transcriptional activation by RXR homodimers is repressed by the presence of RARs (Mangelsdorf, 1991; Lehmann, 1992; Chen, 1995). The DR1 element is also the target of a number of orphan nuclear receptors, including COUP-TF, PPAR, ARP-1 and HNF4 (Rottman, 1991; Kliewer, 1992; Keller, 1993). It was recently shown that the orphan receptor COUP-TF can function as a negative regulator of RA action on a DR1 element via competitive DNA binding (Cooney, 1992; Tran, 1992).

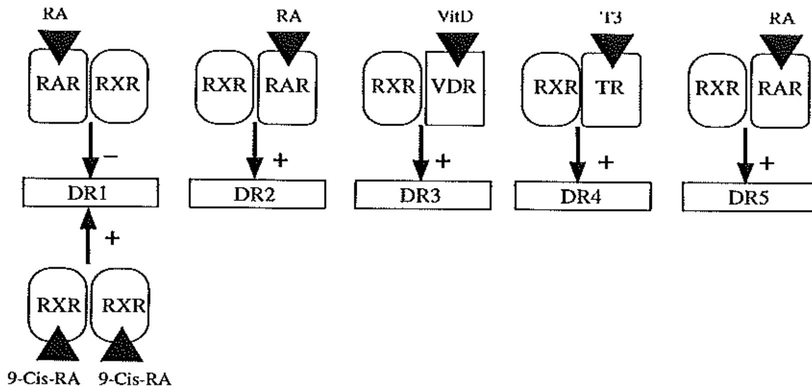

Fig. 5: Nuclear hormone receptors bind their response elements as dimers. RXR acts as a promiscuous heterodimeric partner in nuclear receptor function. The 5'/3' binding polarity of the RAR/RXR dimer on the DNA is represented as 5' = left and 3' = right. Response elements are usually configured as a direct repeat (DR) of the halfsite consensus sequence PuGGTCA, separated by a specific spacer length. RXRs can also bind as homodimers to a DR-1 element in the presence of 9-cis RA. Both activation and repression are observed on DR-1 elements.

Since the non-steroid nuclear receptors bind to their response elements as heterodimers, and the response elements (in general) consist of a 5' and a 3' half-sites, the possibility exists that binding occurs with a specific polarity. Indeed it has been established that RXR always

occupies the 5' half-site on DR2, DR3, DR4 and DR5 motifs, whereas its heterodimeric partner (RAR, VDR, TR and RAR respectively) occupies the 3' half-site (Kurokawa, 1993; Kurokawa, 1994; Zechel, 1994; Schrader, 1994). The situation is reversed on a DR1 elements. Using synthetic RAR and RXR selective ligands it could be shown that on the DR1 element of the rat CRBP-II promoter the RAR heterodimeric partner is bound to the 5' half-site and RXR to the 3' half-site (Kurokawa, 1994). This reversed polarity leads to repression rather than activation of the promoter and it inhibited RXR homodimer mediated activation from this promoter element.

Element	Gene	Sequence	Reference
DR-1	rCRBP-II	acAGGTCAcAGGTCAcAGGTCAcAGTTCAtt	(Mangelsdorf, 1991)
	mCRABP-II	gaAGGGCAgAGGTCAca	(Durand, 1992)
	hApoA1	gcAGGGCAgGGGTCAag	(Kliewer, 1992)
	cOVAL	tgGTGTCAaAGGTCAaa	(Cooney, 1992)
	rPEPCK	caCGGCCAaAGGTCAtg	(Lucas, 1991)
	rACO	ccAGGACAaAGGTCAcg	(Kliewer, 1992)
	HBV	cgGGGTAAaGGTTCAgg	(Islam, 1993)
	mHHC1	tgAGGTCAgGGGTGGgg	(Nagata, 1992)
DR-2	rCRBP-I	gtAGGTCAaaAAGTCAga	(Husmann, 1992)
	mCRBP-I	gtAGGTCAaaAGGTCAga	(Smith, 1991)
	mCRABP-II	ccAGTTCAccAGGTCAgg	(Rottman, 1991)
	hApoA1	agGGGTCAagGGTTCAgt	(Durand, 1992)
	mVL30-1	aaAGTTCAtgTTTTCAca	(Islam, 1993)
	mVL30-2	tgGGGTGAaaAGTTTAgg	(Islam, 1993)
DR-5	hRARb2	agGGTTCAccgaaAGTTCAct	(de The, 1990)
	mRARb2	agGGTTCAccgaaAGTTCAct	(Sucov, 1990)
	mRARa2	cgAGTTCAgcaagAGTTCAgc	(Leroy, 1991)
	hRARa2	gaGGTTCAgcgagAGTTCAgc	(Leroy, 1991)
	hRARg2	ccGGGTCAggaggAGGTGAgc	(Lehmann, 1992)
	hADH3	agGGGTCAttcagAGTTCAgt	(Duester, 1991)
	mCP-H	gcAGGTCActgacAGGGCAta	(Munoz-Canoves, 1990)
	hGa1	caAGGGCAggagaAGGTCAga	(Costa-Giomi, 1992)
	hMGP	aaGGTTCAcctttTGTTCAcc	(Cancela, 1992)
	mHoxa1	caGGTTCAccgaaAGTTCAag	(Langston, 1992)
	mHoxd4	taAGGTGAaatgcAGGTCAca	(Popperl, 1993)
	hCMV-IEP	taAGGTCAtgtcaTGGGCAta	(Ghazal, 1992)
ER-8	mgF-crystallin	agTGACCCttttaaccAGGTCAgt	(Tini, 1993)
	hMCAD	atTGACCTttctctccGGGTAAag	(Raisher, 1992)
Palindrome	TREpal	tcAGGTCATGACCTga	(Umesomo, 1988)
	bGH	ggGGGACATGACCCca	(Williams, 1992)
	hOST	ctAGGTGAcTCACCGgg	(Schule, 1990)
	xVitA2	tcAGGTCAcagTGACCTga	(de Verneuil, 1990)
Complex	rGH	aaAGGTAAgatcaGGGACGTGACCTca	(Umesomo, 1988)
	mLamB1	gaGGTGAgctaGGTTAA(N13)GGGTCAac	(Vasios, 1991)
	hOXY	atTGGTCA(N14)GGGTCA(N47)GGGTCAAGGTCAcc	(Richard, 1991)

Fig. 6: Compilation of retinoic acid response elements (RAREs) found in various RA responsive target genes. RAREs consist of at least two halfsites of consensus sequence PuGGTCA, found in various configurations. RAREs binding RAR/RXR heterodimers exist as direct repeats with 1, 2 or 5 bp spacing, as inverted repeats (palindromes) or as everted repeats with no or 8 bp spacing, but other configurations have been found as well.

An overview of the various retinoic acid response elements is given in Figure 6. Looking at this list, the presence of a large number of genes involved in the transduction of the retinoid signal draws the attention. The retinoid signalling pathway appears to be subject to autoregulation by positive and negative feedback mechanisms at many levels. All three RAR genes contain a RARE in one of their two promoters (de The, 1990; Sucov, 1990; Leroy, 1991; Lehmann, 1992) and autoinduction of RAR expression could result in an amplification of the signal. RA induction of the genes for CRBP-I and alcohol dehydrogenase type 3 (ADH3) may enhance RA synthesis (Smith, 1991; Duester, 1991). Upregulation of CRABP-II (Durand, 1992; McGregor, 1992; Astrom, 1995) and possibly CRABP-I (Leonard, 1995; Kleinjan, 1997) may lead to an increase in RA catabolism and a concomitant decrease of the RA response.

Genetic studies on the retinoid receptors.

The development of the technique of gene targetting in mice by homologous recombination in embryonic stem (ES) cells has made it possible to study the function of a specific gene in its natural, complex situation *in vivo* (Thomas, 1987). In this method, the coding sequences of one allele of the gene of interest are disrupted in ES cells in such a way that no functional transcript is produced from it, and hence no protein is made. When introduced into the inner cell mass of a blastocyst these ES cells can contribute to the resulting organism, including the germ cells. Subsequent breeding of animals that transmit the mutation can result in the generation of mice that are homozygous for the null allele of the studied gene. This technique has been used to study the functions of the retinoid receptors. Null mutations of RARα, RARß and RARγ, as well as isoform specific knock-outs of RARα1, RARß2/4 and RARγ2 have been generated (Li, 1993; Lohnes, 1993; Lohnes, 1994; Lufkin, 1993; Luo, 1995; Mendelsohn, 1994). The RARα and RARγ null mutants display some of the defects of the postnatal vitamin A deficiency (VAD) syndrome, including poor viability, growth deficiency and male sterility, as well as some congenital malformations. Surprisingly, the other RAR knock-out mice appear normal. However, when these mice were crossed with each other in various combinations, thus creating double knock-out mice, most of them exhibited a dramatically reduced viability and specific embryonic defects. Almost all of the malformations of the fetal VAD syndrome are found in the different RAR double mutants (Kastner, 1995; Lohnes, 1994; Mendelsohn, 1994). In addition some developmental abnormalities not found in VAD embryos also appear in the RAR double mutants. This is most probably due the fact that it is very difficult to create a state of severe vitamin A deficiency for the embryos by dietary deprivation of the mothers, because complete vitamin A deficiency is not compatible with pregnancy. Thus, when embryos are produced from VAD mothers there must be some residual vitamin A present. By grouping the malformations produced in the various RAR double null mutants together one can recapitulate the fetal VAD syndrome (Kastner, 1995). Since the RA receptors have RA as their specific ligand, these results indicate that RA is the active vitamin A derivative *in vivo* and that its effects are mediated by the RARs.

Null mutations have also been generated for the RXRα and RXRß genes. However, the RXRs act as heterodimeric partners in a number of different signalling pathways, making it therefore difficult to ascribe a specific phenotype seen in a knock-out mouse to one particular pathway. RXRα deficient mice display eye and heart abnormalities and they die from cardiac failure around 14.5 days of embryonic development (Dyson, 1995; Kastner, 1994; Sucov, 1994). RXRß null mutants are viable and appear normal, but males are sterile due to abnormal sperm maturation and release (Kastner, 1996). Double null mutants between RXRα and different RARs show an increase in the severity of certain abnormalities and leads to new

abnormalities in some combinations. The synergistic effects observed in these double knock-out mice suggest that indeed RXR/RAR heterodimers are the functional units for the transduction of the RA signal *in vivo*. Ultimately, it may be possible to identify the predominant RXR/RAR heterodimer involved in any given process by the generation and detailed analysis of all combinations of retinoid receptor knock-out mice, but this approach will be severely hampered by the apparent redundancy present in the retinoid receptor family, as evident by the lack of phenotype in many RAR single knock-out mice (Mendelsohn, 1994; Thomas, 1993).

Redundancy of the RARs is also found in EC cells. In RAC65 cells, a P19 derivative which carries a dominant-negative RARα allele, RA-induced expression of HoxA1 is blocked. Expression is restored by transfection of any of the three RAR genes, and not just by RARα (Pratt, 1993). Similarly, a RARγ null mutant F9 cell line, which shows aberrant differentiation and reduced expression of RA-induced genes, can be rescued by overexpression of RARα, and partially rescued by overexpression of RARß (Taneja, 1995).

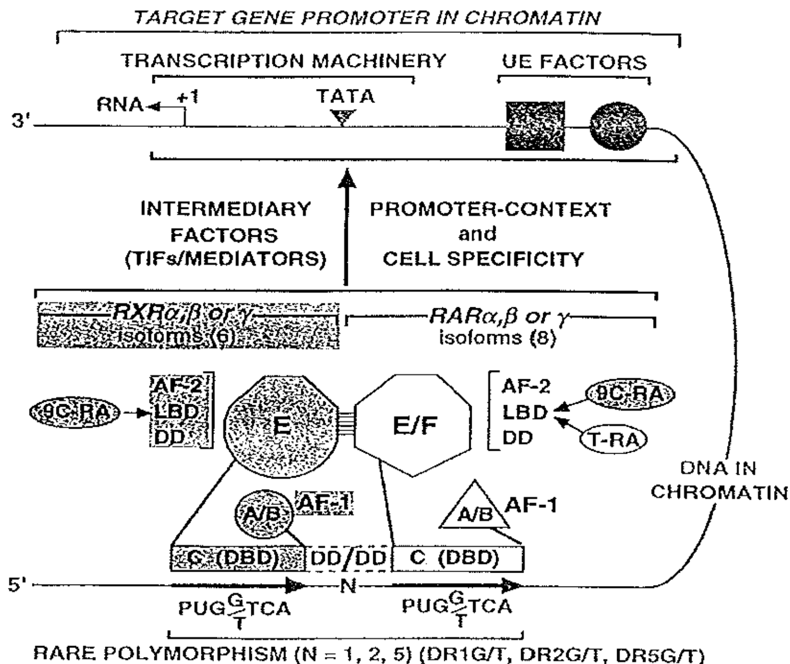

Fig. 7: Model showing the complex of an RAR/RXR heterodimer on a retinoic acid response element (RARE) in the promoter region of a target gene. The model integrates the current knowledge on target gene activation by the retinoic acid receptors. RARs can bind both all-trans RA and 9-cis RA, whereas RXRs only bind 9-cis RA. The receptors consist of six functional domains, A to F. The DNA binding domain (DBD) is located in the C domain, while the E domain contains the dimerisation domain (DD), the ligand binding domain (LBD) and a ligand dependent activation domain (AF-2). The RA receptors bind to response elements consisting of a direct repeat (DR) of at least two half-sites, with halfsite recognition sequence PuGGTCA and a spacing of 1, 2 or 5 basepairs between the halfsites. Ligand activated RAR/RXR heterodimers interact with the trasncription initiation complex either directly or via transcription intermediary factors (TIFs). (Adapted from (Chambon, 1996)).

I.3: CRABP-I and the cellular retinoid binding proteins.

Having introduced the mechanisms of the RA signal transduction pathway, the metabolism of RA, and the nuclear RA receptors and their response elements in the previous paragraphs, the final paragraph of this introductory chapter will focus on the intracellular retinoid binding proteins, and in particular on CRABP-I.

Protein structure and evolutionary conservation of CRABP-I.

CRABP-I is a small intracellular RA binding protein. It belongs to a family of cellular lipid binding proteins that bind small hydrophobic molecules such as retinoids and fatty acids. This family of proteins includes CRABP-I and II, CRBP types I and II, the myelin protein P2, adipocyte P2, and several other fatty acid binding proteins (Bass 1993; LaLonde, 1994). All members of the cellular lipid binding family are in the molecular weight range of 15-16 kD. CRABP-I has 136 amino acids, CRABP-II has an extra amino acid at position 116 relative to CRABP-I and thus consists of 137 residues (Giguere, 1990). Two CRABP isoforms, called xCRABPa and xCRABPb, have been identified in *Xenopus laevis* (Dekker, 1994; Ho, 1994). Based on sequence comparison both xCRABPs most closely resemble CRABP-II. However, xCRABPa is 8 amino acids longer at its C-terminus than CRABP-II from other species.

The CRABPs are highly conserved proteins through evolution. Mouse CRABP-I is identical at the amino acid level to rat and bovine CRABP-I (Sundelin, 1985; Rajan, 1991; Stoner, 1989; Vaessen, 1990). They differ from human CRABP-I at only one of the 136 residues, a proline to alanine substitution at position 86 of the protein (Astrom, 1991). In chapter 6 of this thesis the cloning and sequencing of CRABP-I from chicken and pufferfish is described. Having 94% and 86% homology with mouse CRABP-I respectively, the protein sequences of these CRABP-I homologues confirmed the strong evolutionary conservation of the protein. Figure 3 in Chapter VI shows an alignment of all known CRABP-I amino acid sequences. For comparison, the sequences of human and mouse CRABP-II have been added to this list.

CRABP-II is also strongly conserved, with a 94% protein sequence identity between human and mouse (Astrom, 1991; Giguere, 1990). The amino acid identity between the two mammalian CRABPs (i.e. CRABP-I and -II) is 73%. The differences between CRABP-I and II are found mainly in residues with hydrophilic side chains, which are most likely present at the surface of the protein. The high degree of conservation of the CRABPs indicates that the structures of both proteins have been under strong selective pressure. The homology to the CRBPs is much lower. Within the cellular lipid binding protein family the CRABPs are as equally related to the myelin protein P2 and adipocyte P2 as to the CRBPs (40% and 37% respectively). These sequence similarities could mean that the lipid binding protein family has evolved from a common ancestor gene.

Both CRABPs bind all-trans RA with high, but different affinities. CRABP-I has the highest affinity for RA of any known protein in the cell. The affinity of CRABP-I for all-trans RA is stronger than can be measured exactly by existing fluorescence techniques, but the apparent equilibrium dissociation constant (K_d) of CRABP-I for RA is approximately 0.4 nM . The K_d of CRABP-II is around 2 nM, and thus about five fold higher than that of CRABP-I (Norris, 1994). The affinity of the CRABPs for RA appears to be higher than that of the RA receptors, but since different methods have been used to measure the affinities a direct comparison cannot be made (Allenby, 1993; Norris, 1994). The K_ds of the RARs have been determined at around 1 nM (Napoli 1996). The CRABPs show a strong preference for the all-trans form of RA, since 9-cis RA and 13-cis RA binding occurs with much lower affinity than

all-trans RA (Fiorella, 1993; Norris, 1994). They do not bind retinol, retinal or retinyl esters (Fiorella, 1993). The natural retinoic acid metabolites 4-hydroxy RA, 4-oxo RA, 18-hydroxy RA and 3,4-didehydro RA, as well as synthetic RA analogues with a modified ring structure, are bound by both CRABPs (Fiorella, 1993).

The three-dimensional structures of CRABP-I and CRABP-II are very similar to each other, and very similar to the structure displayed by other members of the cellular lipid binding protein family (Kleywegt, 1994; Jones, 1988; Sacchettini, 1989/1989; Cowan, 1993; Newcomer, 1984). Both proteins form a compact, single domain structure consisting of two antiparallel ß-sheets that form an orthogonal ß-barrel (Fig. 8). The sheets are built up from 10 ß-strands connected through short reverse turns, except for the linkage between the first and second strand, which consists of a pair of antiparallel α-helices. The protein forms a flattened structure, referred to as a "ß-clam" structure, with dimensions of 40x40x30 Å. When bound, the all-trans RA is sandwiched between the two ß-sheets with its cyclohexenyl ring exposed to the solvent and the carboxyl group deeply buried in the binding pocket, where it interacts with specific amino acid side chains (Kleywegt, 1994). The helix-turn-helix motif is located at the ligand entrance. Electrostatic interactions are thought to play major roles in binding of RA by the CRABPs, because both proteins bind RA with high affinity, but do not bind retinol or retinal.

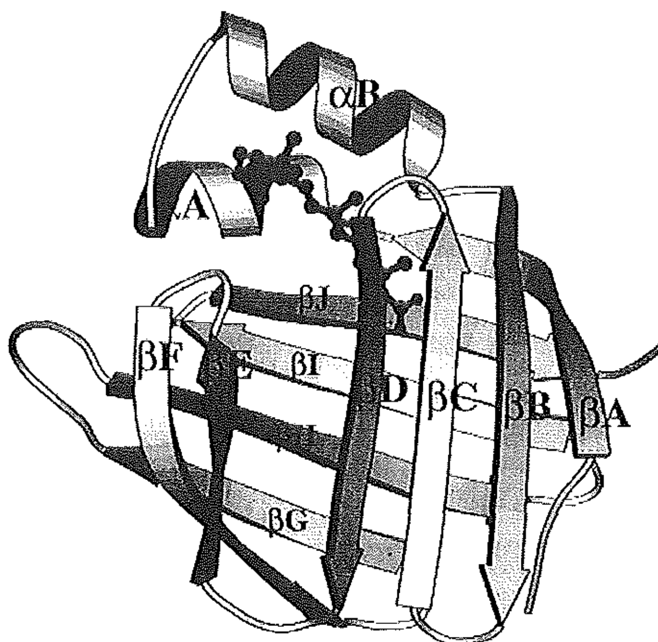

Fig. 8: Ribbon representation of the protein structure of the members of the small intracellular lipid binding protein family. The crystal structure of holo-CRABP-II is shown (Kleywegt, 1994). The 10 β strands are indicated as βA to βJ, and the two a helices as αA and αB. A retinic acid molecule is drawn in its binding cavity.

Crystallographic data indicate a direct interaction occurs between the carboxyl moiety of RA with Arg-131 of CRABP-I (Arg-132 in the case of CRABP-II) and an indirect interaction is thought to form with Arg-111 via bound water (Kleywegt, 1994). These two arginine residues, which are evolutionary conserved (see Chapter VI, this thesis), are critical for RA binding by the CRABPs. Site-directed mutagenesis of either of these residues in CRABP-I, changing the arginine to glutamine, drastically reduces all-trans RA binding (Chen, 1995). Mutation of Arg-132 to either alanine, glutamine or methionine, and of Arg-111 to methionine or alanine showed

the importance of both arginines for RA binding of CRABP-II (Chen, 1995; Wang, 1997). The cellular retinol binding proteins (CRBPs), which bind both retinol and retinal but select against RA, have glutamines at the positions of the conserved arginine residues in the CRABPs.

The genes for CRABP-I, CRBP-I and CRBP-II have all been mapped to chromosome 9 in the mouse (Demmer, 1987; Geurts-van Kessel, 1991), and the CRABP-II gene to chromosome 2 (Giguere, 1990). CRBP-I and II are also linked in humans, where they are located on chromosome 3. They are separated by only three centimorgans, consistent with the idea that duplication of one CRBP led to the existence of the pair (Demmer, 1987). CRABP-I however, is not linked with the CRBP genes in humans and is found on chromosome 15 (Vaessen, 1991), while human CRABP-II is located on chromosome 1 (Ong, 1994).

The murine gene for CRABP-I

The genomic structure of the CRABP-I gene from the mouse has been determined (Wei, 1990). The gene spans 10 kb, and consists of four exons of 130, 90, 60 and 120 basepairs respectively. The exons are spaced by introns of 0.5kb, 3.5kb and 5.5 kb. The gene encodes an 800 bp mRNA. The transcriptional start site has been mapped to the G-residue at position -95 relative to the translational start site. The promoter region of the gene lacks TATA and CAAT boxes, but in the 300 bp immediately preceding the transcriptional start five recognition sites for the transcription factor Sp1 are located. The sequence of a three kb region upstream of the gene has been determined, but no obvious transcription factor binding sites were found. Figure 9 shows a simplified map of the murine CRABP-I locus.

Fig. 9: The murine gene for CRABP-I. The gene spans about 10 kb and consists of four exons (boxes indicated by Roman numerals; the filled space indicates the coding region), spaced by introns of 0.5kb, 3.5kb and 5.5kb. E = EcoRI, H = HindIII, S = SalI, X = XhoI.

Expression patterns

The expression patterns of the various proteins involved in the retinoid signalling pathway(s), i.e. the RARs/RXRs and the retinoid binding proteins, have been studied extensively. Both RNA *in situ* hybridization and protein immunohistochemistry have been used to show that almost all of them exhibit restricted patterns of expression during embryonic development, as well as in the adult.

RARα shows the least specific pattern of expression, as it is almost ubiquitously expressed throughout embryogenesis, as well as in adult tissues. Together with the fact that the RARα1 promoter exhibits characteristics of a housekeeping gene promoter, this has led to the suggestion that RARα is a housekeeping gene. (Brand, 1990; Leroy, 1991). However, in early embryos certain tissues do not express RARα; for instance, in a E9.5 embryo, RARα expression cannot be detected in a region of the CNS which includes the forebrain, the midbrain, and

rhombomeres 1 to 3 of the hindbrain (Ruberte, 1991).

Unlike RARα, RARß and RARγ exhibit very specific patterns of expression during embryonic development, which are in general mutually exclusive (Dolle, 1990; Ruberte, 1991). RARγ is mainly localised in precartilaginous condensations of mesenchyme, and continues to be expressed during cartilage differentiation and in mature cartilage, being lost from these sites only when ossification starts (Ruberte, 1990). RARß is predominantly expressed in the developing CNS, and in the mesenchyme destined to form muscle (Ruberte, 1990; Ruberte, 1993; Ruberte, 1992). The mutual exclusiveness of RARß and RARγ is clearest in the developing hand or foot plate, where RARg is expressed in the skeletal elements of the digits and RARß in the interdigital mesenchyme (Dolle, 1990; Mendelsohn, 1991). In the adult RARa shows an ubiquitous expression pattern, while RARß is predominantly found in kidney, adrenals, spinal cord, muscle and prostate, with lower levels in several other organs. RARγ has an even more restricted tissue distribution in the adult, with expression found only in skin and lung.

Like RARα, RXRß expression is ubiquitous in both adult and embryo. RXRα shows a restricted expression pattern in adult and embryonic tissues, while the RXRγ tissue distribution is very similar to that of RARß. Virtually every tissue expresses at least one RAR and one RXR (Zhang, 1992a). A detailed description of the expression patterns of the RARs and the RXRs can be found in the original papers (Dolle, 1989; Rochette-Egly, 1994; Ruberte, 1990; Ruberte, 1992).

The expression patterns of the various intracellular retinoid binding proteins, CRABP-I and -II, CRBP-I and -II are highly spatio-temporally restricted, both at embryonic stages and in the adult. In adult tissues both CRBP-I and CRABP-I appear to be rather ubiquitously expressed at a low basal level. Only in certain RA sensitive tissues such as the eye and testis are CRABP-I levels more elevated. On the other hand, expression of CRBP-II and CRABP-II is highly restricted in the adult. CRBP-II is expressed solely in the absorptive cells of the intestine (the enterocytes), where it functions in the absorption of retinol and retinal from the diet (Ong, 1984). It is most abundant in the cytosol of the jejunum, where it comprises about 1% of all soluble protein of the mature enterocyte. CRABP-II is strongly expressed in the epidermis of the skin, in the fibroblasts and keratinocytes (Sanquer, 1994; Gendimenico, 1995; Elder, 1992; Elder, 1993). Some of these studies also report the presence of CRABP-I in the skin, presumably in the melanocytes (Sanquer, 1994).

As mentioned, the expression patterns of CRABP-I, CRABP-II and CRBP-I are highly spatiotemporally specific during embryonic stages (Dolle, 1989; Dolle, 1990; Ruberte, 1990; Ruberte, 1992). The second retinol binding protein, CRBP-II, is not expressed at embryonic stages. It has been noted by a number of authors that the expression of CRABP-I appears to coincide with those structures that are particularly sensitive to excess of vitamin A, whereas CRBP-I expression seems to coincide more with the tissues that are vulnerable to deficiency of vitamin A. The expression patterns of CRABP-I and CRBP-I are indeed non-overlapping in many embryonic structures (Dencker, 1991).

The sites where RA accumulates in the embryo have been determined in an experiment in which a radioactively labelled synthetic derivative of RA, [^{14}C]TTNPB, was administered to pregnant female mice (Dencker, 1990). The radioactively labeled RA analogue appeared to accumulate in the CNS, the craniofacial area, the branchial arches, dorsal root ganglia, in cells along the gut and the major vessels of the trunk region. In the CNS the radioactive RA analogue was found in the outer layer of the midbrain, hindbrain and neural tube. It seems reasonable to assume that endogenous RA would localise in the same sites as the exogenously added [^{14}C]TTNPB. These sites of RA accumulation are exactly the structures in the embryo that

express CRABP-I, suggesting that CRABP-I is specifically expressed in tissues were RA is active, or that, due to the presence of CRABP-I, RA is specifically sequestered in those tissues.

The expression pattern of CRABP-II in the embryo is less suggestive of a particular function. CRABP-II is more widely expressed than CRABP-I, but its level of expression is generally lower. CRABP-I and CRABP-II show overlap in many of their expression sites. However, there are some tissues in which CRABP-II is expressed and CRABP-I is not, including the pharyngeal pouches, oesophagus, tracheal epithelium and the liver (Ruberte, 1992). CRABP-II is expressed throughout the thickness of the neural tube, whereas expression of CRABP-I in the CNS is restricted to the mantle layer. Both CRABPs are expressed in the neural crest cells. Because of its relevance to the work described in this thesis, the expression pattern of CRABP-I in the embryo will be described in more detail below. Detailed descriptions of the expression patterns of CRBP-I and CRABP-II have been described in the original papers (Ruberte, 1990/ 1992/1993).

CRABP-I expression is first seen at the presomite stage (E8) in the future hindbrain neuroepithelium, just before closure of the neural tube (Leonard, 1995). At embryonic day 8.5 (E8.5) to E9 expression of CRABP-I is found in the caudal two thirds of the hindbrain region, in the two caudal-most of the three prerhombomeres that can be recognised at this stage. Expression is also found in the migrating neural crest cells. In E9.5 embryos eight rhombomeres are discernable in the hindbrain. The CRABP-I positive area of the hindbrain neuroepithelium is now identifiable as rhombomeres (rh) 2, 4, 5 and 6. The expression level is low in rh2, high in rh4, 5 and 6 and trailing off in rh7 and more caudally (Maden, 1991). At this stage CRABP-I is present throughout the thickness of the neuroepithelium in these specific rhombomeres. Expression is also found in the outer layer of the roof of the midbrain. The forebrain does not express CRABP-I at any developmental stage. In the spinal cord only the cells in the mantle layer of the neural tube express CRABP-I. CRABP-I is also expressed by all neural crest cells. Neural crest cells migrating from the hindbrain express CRABP-I at a level according to the rhombomere from which they derive, e.g. at low level in the neural crest cells coming from rh2, and at high levels in the neural crest cells from rh4 and 6. Neural crest cells from rh2 migrate into the first branchial arch, those from rh4 go to the second branchial arch, and the crest cells from rh6 migrate into arch 3. This results in the mesenchyme of arch 1 being weakly CRABP-I positive, and of arches 2 and 3 being more strongly positive. CRABP-I is also strongly expressed in the frontonasal mesenchyme. This mesenchyme is derived from midbrain crest, at least in the chick embryo (Lumsden, 1991). In addition expression is found in the neural crest cells that migrate laterally of the neural tube.

At 10.5 days p.c. the expression throughout the thickness of the rhombomeres fades, to be replaced by expression in the mantle layer of the whole rhombencephalon. Expression in the midbrain roof and the mantle layer of the spinal cord remains. Expression in the branchial arches also disappears around 10.5 days p.c.. The frontonasal mesenchyme remains positive. The dorsal root ganglia of the trunk as well as the cranial nerves are strongly positive. The limb buds have appeared at this stage and are weakly CRABP-I positive in the progress zone underlying the apical ectodermal ridge (AER) (Dolle, 1989). The expression level of CRABP-I in the embryo is highest in the period around 11 days p.c.. After this stage the level of expression gradually decreases, until expression has virtually disappeared at 16 days p.c..

The expression pattern of CRABP-I has also been studied in detail in the chicken embryo (Vaessen, 1990; Maden, 1991/1994). Small differences in their expression patterns of CRABP-I appear to exist between the mouse and the chicken. In the hindbrain of the chicken CRABP-I expression is high in rh4, low in rh5, and then present in rh6 and caudally (Maden, 1991). In contrast to the situation in the mouse the expression in rh2 is missing and the expression in rh5

is much lower. In addition, the otic vesicle of the chicken expresses CRABP-I, while this has not been reported for the mouse embryo.

Regulation of retinoid binding proteins.

The regulation of the gene for CRABP-I during mouse embryonic development forms the main theme of this thesis. During the course of this work a number of other studies concerning the regulation of CRABP-I have been reported, mainly performed on cell lines. The first 3 kb upstream of the gene have been sequenced (Wei, 1991). The putative promoter region lacks TATA and CAAT boxes, but it has a high GC content in the region around the first exon of the gene. Five Sp1 recognition sites are located in the 300 basepairs immediately upstream of the transcriptional start site. However, this immediate upstream region by itself is insufficient to direct expression of the gene in mouse embryos (Kleinjan, 1997). Furthermore, in the cell line MES-1, which was obtained by differentiating P19 EC cells through aggregation in the presence of DMSO (Mummery, 1986), and which expresses CRABP-I at a high level, the same region was also unable to induce expression of a CAT reporter gene (Chapter III, this thesis). In an early report on CRABP gene regulation a very incomplete reproduction of the CRABP-I expression pattern was obtained in transgenic mice with a 3 kb upstream region linked to a Hox1.3 promoter-LacZ fusion (Wei, 1991). The reported expression pattern of the LacZ reporter gene in that study was vague and unconvincing, and was presumably the result of the combination of part of a CRABP-I regulatory region with the Hox1.3 (Hox A5) basal promoter. In this thesis we show that only the elements that direct expression in neural tissue in the embryo are located within this 3 kb upstream region, and that other elements driving CRABP-I expression are located downstream of the promoter region.

In various cell lines a number of factors have been shown to be able to influence expression of CRABP-I. These include FGF2, BMP2, BMP4 and retinoic acid (Chen, 1996; Wei, 1989). In Balb 3T3 cells, which normally express CRABP-I at low levels, FGF-2 (basic fibroblast growth factor) treatment increased the level of CRABP-I. The upregulation by FGF-2 appeared to occur post transcriptionally by increasing mRNA stability. Treatment with bone morphogenetic proteins, BMP-2 or BMP-4 reduced the level of CRABP-I, as well as the levels of CRABP-II and RARß1/ß3 transcripts (Means, 1996). The reports on the inducibility of CRABP-I by RA are in apparent disagreement with each other. Downregulation of CRABP-I expression by RA has been observed in MES-1 cells (Vaessen, 1991), as well as in the F9 embryonal carcinoma cell line (Stoner, 1989). On the other hand, upregulation of CRABP-I expression was found in P19 cells (Wei, 1989). Addition of sphinganine, a mitogen that has been shown to activate transcription factor AP-1 (Su, 1994), could enhance this upregulation in P19 cells (Wei, 1995). The presence of a promoter fragment that contains a putative AP-1 binding site was shown to be required for this effect (Wei, 1995). AB1 EC cells express CRABP-I at low level, and expression is induced to much higher levels by RA treatment. Remarkably, this induction is only seen at low RA concentrations (10^{-9} M), and is almost extinguished at higher RA concentrations (10^{-7} M).

In addition, demethylation of the flanking regions of the transcriptional start site of the gene has been associated with activation of the gene (Wei, 1994). This region is hypermethylated both in untreated P19 cells and in adult mouse tissues, where expression of CRABP-I is low. During early stages of mouse embryogenesis, when CRABP-I expression is high, a demethylation of the CRABP-I promoter region is found (Wei, 1994). Treatment of P19 cells with 5-azacytidine (5-azaC), a DNA methylation inhibitor (Glover, 1987), results in enhancement of the upregulation of CRABP-I by RA by approximately five fold. Partial

demethylation of the 5' flanking region of the CRABP-I gene is observed in the treated P19 cells.

A number of studies have looked at the effect of RA on CRABP-I expression in embryos. In one of those studies an upregulation and anteriorisation of CRABP-I expression was found (Leonard, 1995). However, other studies did not observe such an effect (Harnish, 1992), but this may be due to the exact timing of the RA treatment. Chapters IV and V of this thesis describe the analysis of the regulation of CRABP-I in transgenic mouse embryos. Interestingly, one the elements found to be involved in the transcriptional regulation of the gene during mouse development is a putative retinoic acid response element (RARE).

While at the outset of this thesis little was known about the regulation of the CRABP-I gene, the CRABP-II, CRBP-I and CRBP-II genes were known to be transcriptionally regulated via high affinity RA response elements. Different types of RAREs are contained in the promoters of these genes, but all three genes have been shown to be inducible by RA (Haq, 1988; Giguere, 1990; Smith, 1991;Durand, 1992).

The CRABP-II gene is induced by at least 50-fold upon treatment of F9 cells with retinoic acid (Giguere, 1990). The promoter for mouse CRABP-II contains a typical TATA-box and a GC-rich region with putative Sp1, AP-1 and AP-2 binding sites. In addition two RAREs, RARE1 and RARE2, are located further upstream, which cooperate in the transcriptional activation by RARs and RXRs in P19 EC cells (Durand, 1992). RARE1 and RARE2 are direct repeats of two motifs separated by 2 bp (DR2) and 1 bp (DR1) respectively. RAR/RXR heterodimers binding to these motifs have been shown to be responsible for CRABP-II promoter transactivation. Topical application of RA to human skin *in vivo* and addition of RA to cultured human skin fibroblasts also increased CRABP-II transcription (Astrom, 1991). In several hyperproliferative conditions of the skin a highly induced expression of CRABP-II is observed, while CRABP-I expression is reduced (Didierjean, 1991; Busch, 1992). The upregulation of CRABP-II in these skin disorders may be the result of an increased availability of RA, resulting from the downregulation of CRABP-I. Comparison of the promoters of the mouse and the human CRABP-II genes revealed that the DR-2 RARE is absent in the human CRABP-II promoter. The DR-1 RARE has been conserved and an extra DR-1 is present in the same region, but both these RAREs appear to be non-functional in all human cell lines tested. Instead a far upstream DR-5 RARE mediates the induction of human CRABPII transcription by RA (Astrom, 1994). Like the CRABP-II gene, the genes for CRBP-I and CRBP-II are also induced by RA treatment (Haq, 1988; Wei, 1989). The CRBP-I gene has a TATA-less promoter, but a cluster of Sp1 factor binding sites is present in the immediate upstream region of the gene (Jones, 1985; Kadonaga, 1987). In addition, it contains a RARE of the DR2 type, which is predominantly activated by RAR/RXR heterodimers (Husmann, 1992; Smith, 1991). The gene for CRBP-II contains a TATA-box and a CAAT-box, and it also contains a special type of response element consisting of five nearly perfect tandem repeats of the AGGTCA half-site sequence spaced by single C nucleotides (Demmer, 1987). This response element has been termed an RXRE since it is only transactivated by RXR homodimers which form in the presence 9-cis RA, and not by RAR/RXR heterodimers (Mangelsdorf, 1991).

The function of CRABP-I

The cellular retinoic acid binding proteins (CRABP-I and II) are likely to be important in regulating the availability of RA to the nuclear receptors, but the mechanism by which they perform this task remains enigmatic. A number of potential roles for the CRABPs have been

proposed. These centre around the possibilities that they act simply as a cytoplasmic buffer for RA, or, more actively, as a modulator of RA metabolism, thus protecting cells from excess RA reaching the receptors (Donovan, 1996). Alternatively their role may be to sequester RA in specific cells under deficiency conditions, or, as a nuclear localisation of CRABP has been detected in certain cells, they could form a nuclear translocation system (Gustafson, 1996). Two systems have been used to further assay the function of CRABP-I: teratocarcinoma cell lines and genetically modified mice.

Retinoic acid and CRABP-I in mouse teratocarcinoma cell lines.

Retinoids can influence the differentiation and proliferation of many different cell types (Sporn, 1994). In general retinoids inhibit proliferation and induce differentiation. They can induce the differentiation of human promyelocytic leukaemia HL60 cells into mature granulocytes (Breitman, 1980) and inhibit the proliferation of S91 melanoma cells (Lotan, 1980). In contrast, retinoids are found to inhibit the terminal differentiation of human keratinocytes (Fuchs, 1981) Because of their anti-proliferative and differentiation-inducing activity retinoids have been tested as therapeutic agents against cancer. RA also induces the differentiation of several mouse teratocarcinoma cell lines (Strickland, 1980). The system of teratocarcinoma cell lines has been used extensively to study the effects of RA on differentiation.

Mouse teratocarcinoma cell lines resemble the pluripotent cells of the inner cell mass of the early embryo (Martin, 1980). They provide an important model system, in which lineage determination in early mouse development can be studied. The F9 teratocarcinoma cell line, grown in monolayer culture can be induced to differentiate into primitive endoderm by treatment with a physiological concentration of retinoic acid (RA) and into parietal endoderm, an extra-embryonic cell type in the blastocyst, in response to RA and dibutyryl cAMP (Strickland, 1980). This RA induced differentiation response is synchronous and rapid. Upon differentiation of teratocarcinoma cells many genes are differentially expressed. The RA treatment results for instance in the appearance of high levels of lamininB1 and collagen IV, two parietal endoderm-specific genes (Hosler, 1989; Wang, 1989), while it also causes a morphological change in the F9 cells.

CRABP-I mRNA is constitutively expressed in F9 cells, whereas CRABP-II is undetectable by Northern blot analysis (Stoner, 1989, Giguere, 1990). RA treatment of F9 stem cells results in a 2 to 3 fold reduction of CRABP-I mRNA and a 50 fold increase of CRABP-II mRNA by 48 hours after treatment. Treatment with RA and dibutyryl cAMP gives a 20 fold increase of CRABP-I mRNA (Stoner, 1989). Stably transfected F9 cells which overexpress CRABP-I have been shown to require a higher concentration of RA to initiate differentiation, whereas F9 cells having a reduced CRABP-I level due to the stable transfection of a CRABP-I antisense expression vector, respond to a lower RA concentration (Boylan, 1991). This observation is consistent with the hypothesis that CRABP-I functions as an intracellular RA buffer. In addition, the level of CRABP-I also influences the metabolism of RA in those cells. F9 stem cells readily metabolise all-trans RA to more polar compounds such as 4-oxo-retinoic acid and 4-oxo-16-hydroxy retinoic acid. The metabolic half-life of RA is reduced after pre-treatment of the F9 cells with RA, suggesting that the enzymes involved in RA metabolism are induced by RA itself (Williams, 1985). As mentioned before, the major metabolic pathway of RA consists of the hydroxylation followed by the oxidation at position C-4 of the cyclohexenyl ring to form 4-oxo-RA (see RA uptake and metabolism). The CRABP-I overexpressing F9 cell line produced higher levels of 4-oxo-retinoic acid and exhibited a shorter intracellular half-life

of (^{3}H)RA as compared with untransfected F9 cells. In contrast, the CRABP-I antisense RNA expressing F9 cell line exhibited an extended intracellular (^{3}H)RA half-life, and produced lower levels of 4-oxo-RA (but higher levels of 13-cis-RA) (Boylan, 1992). These observations suggest that CRABP-I has a role in the metabolism of RA.

Transgenic mice with retinoid binding proteins

A number of studies with genetically altered mice have been done to investigate the function of the retinoic acid binding proteins *in vivo*. These include both the production of transgenic mice expressing CRABP-I under the control of a heterologous promoter, and the knock-out of the CRABP-I and CRABP-II genes through homologous recombination in embryonic stem cells. Although some pathological effects were found, no clear conclusions about the function of CRABP could be drawn from these studies. Transgenic mice that express bovine CRABP-I under control of the human metallothionein IIA promoter showed variable expression of the transgene. Of the six lines obtained, three were normal, one showed retarded growth and two lines produced only female transgenic offspring which were all sterile (Wei, 1991). Directing expression of CRABP-I to the lens, where it is not normally expressed, via the mouse αA crystallin promoter resulted in two phenotypes: defective lens fiber differentiation and pancreatic tumorigenesis (Perez-Castro, 1993). Cataracts, presumably due to impaired cellular differentiation, were found in these animals. However, the expression of γF-crystallin, a RA sensitive gene (Tini, 1993), was not altered, which suggests that the ectopic presence of CRABP-I in the lens did not alter nuclear RA levels. The reason for the pancreatic tumours is unclear, but the transgene was expressed in the tumors. Thus, no clear insight into the function of CRABP-I was obtained through these studies.

Two independent studies have produced mice deficient for CRABP-I via gene targeting (de Bruijn, 1994; Gorry, 1994). No phenotype could be identified in these mice, leading to the conclusion that CRABP-I is not required for normal growth and development. CRABP-II expression was unchanged in these mice. When embryos of the CRABP-I deficient mice were challenged with excess RA at day 8.5 of their development, and examined at day 18.5 of development, no difference was detected in the observed phenotypes between the CRABP-I deficient embryos and their wildtype littermates (Gorry, 1994). This suggests that CRABP-I does not have a unique function in teratogenic resistance or sensitivity, at least not at this specific stage of embryonic development.

Similarly, CRABP-II knock-out mice have been produced. These CRABP-II null mutants do not show any apparent phenotype either, except for the appearance of an additional postaxial digit in the forelimbs of the homozygous mutants (Fawcett, 1995; Lampron, 1995). Thus CRABP-II may have a role in limb development. Mice deficient for both CRABP-I and CRABP-II display the same phenotype as the CRABP-II single null mutant, excluding the possibility of functional redundancy between these two genes. No remaining RA binding activity was detected in whole embryo cytosolic extracts of the double null mutants, showing that lack of CRABP-I and -II was also not compensated by unknown RA binding proteins (Lampron, 1995). The penetrance of the appearance of postaxial digits was higher in the CRABP-I/CRABP-II double null mutants, and their viability appeared to be slightly decreased. However, neither the expression of the RARs, nor the teratogenic susceptibility to RA was changed in the double knock-out mice. Thus, the lack of an apparent phenotype in mice that are homozygously deficient for both CRABP genes was surprising and puts their importance in development and homeostasis into question. However, the high degree of conservation of the

CRABPs and their very strictly regulated patterns of expression during embryonic development suggest an important function for these proteins. This function may be connected with behavioural patterns of the mice, as both CRABPs are expressed in a specific manner in the developing nervous system. Alternatively it has been suggested that they could serve a function which is dispensable in the protected environment of the laboratory, or which gives a slight evolutionary advantage and would thus require many generations to become manifest (Lampron, 1995). Either way, the elucidation of the role of the CRABPs by analysis of phenotypic alterations in genetically modified mice is complicated by these findings.

Outline of the experimental work

The important role of vitamin A in the biology of vertebrate organisms has been known for a long time. The introductory chapter clearly shows that at the molecular level a complex situation underlies the many, widespread effects of vitamin A and its derivatives, most notably retinoic acid (RA). Understanding of the RA signalling pathway(s) has rapidly progressed over the past decade through the identification of the multiple inherent components of the pathways. These components include the multiple biologically active retinoids, their various binding proteins, the nuclear RA receptors, the RA response elements and a still increasing number of accessory proteins. Eventhough much of the basic concept of the RA signal transduction pathway(s0 is now clear, many questions still remain to be answered. One of these questions is how the intracellular RA binding proteins CRABP-I and -II fit into the picture of the molecular mechanisms underlaying RA signalling. Through the work described in this thesis we have tried to increase our knowledge of various aspects of the CRABP-I gene, with the ultimate aim of identifying the function(s) of CRABP-I in the organism.

Even though much was known already about the cellular retinoic acid binding protein type I (CRABP-I) concerning its binding properties, crystal structure and expression pattern in chicken and mouse, the function of the protein was (and unfortunately still is) unclear. While other laboratories were planning to generate mice deficient for CRABP-I, CRABP-II, and by interbreeding for the combination of both CRABP-I and CRABP-II, by employing the technique of gene targetting, we decided to study CRABP-I via careful manipulation of the levels of expression in various tissues during development of mouse embryos. For this a better knowledge of the regulation of expression of the CRABP-I gene was adament. Furthermore we anticipated that identifying the factors involved in regulating CRABP-I expression would tell us more about the regulatory pathways and hierarchies, and the potential feedback mechanisms of retinoid signalling in the developing embryo. During the course of our study the results of the CRABP-I and II knock-out studies became known. Surprisingly, no apparent phenotype could be detected in those mice, at least not under laboratory conditions. In accordance with this, no clinical phenotype is known to be associated with CRABP-I. Yet, the evolutionary conservation of CRABP-I is extremely high, and the gene shows a highly spatio-temporally restricted expression pattern during embryogenesis. The latter observations suggest that CRABP-I does have an important function for the organism, but that we fail to detect this function because at present our techniques and knowledge are insufficient.

Our studies on the CRABP-I gene started with the generation of antibodies against the protein, and its family members CRABP-II and CRBP-I (the cellular retinol binding protein type I). These three members of the retinoid binding protein family are expressed in a specific pattern during embryogenesis, while CRBP-II is not expressed at all during embryonic stages. The specificity and effectiveness of the antibodies was tested first by Western blot analysis and consequently in a study of the expression patterns of these proteins in the developing mouse heart. The results of these studies are described in chapter II. The heart is one of the organs whose development is affected by retinoic acid. To find arguments for (or against) the putative involvement of the CRABPs in heart development we have performed a detailed study of the expression patterns of these retinoid binding proteins in developing mouse hearts. The study shows that the proteins are differentially expressed during the development of the heart. The development of an effective and specific antibody against CRABP-I was also very useful for our studies of the transcriptional regulation of the CRABP-I gene. Eventually, the study of the regulatory mechanisms that direct the specific expression pattern of the gene in the developing

embryo would turn out to form the major part of this thesis. The knowledge of the regulation of the gene obtained by that study now allows us to set up some experiments to study the function of CRABP-I. The initial studies on CRABP-I gene regulation were carried out in cell lines that express CRABP-I at high level. However it soon became clear that these studies, that are described in Chapter III were uninformative and it showed the need to study the regulation of the gene in its endogenous situation, i.e. in the mouse embryo. Thus we have made a detailed study of the regulation of CRABP-I expression during murine embryonic development by generating transgenic mice with genomic constructs from the CRABP-I locus. These experiments are described in Chapters IV and V. In Chapter III we show that the expression pattern of CRABP-I appears to be composed of two different domains, a neural and a mesenchymal/neural crest domain. Expression in these domains is regulated via separate regulatory regions. A more detailed analysis of the regulatory region driving expression in the neural subdomain is performed in Chapter V, which resulted in the identification of the cis-acting elements that form the neural enhancer element of CRABP-I. Some of the transcription factors binding to these elements could be identified. Using the regulatory regions for both subdomains of the total CRABP-I expression pattern, two transgenic mouse lines were generated which have an elevated level of expression of CRABP specifically within its normal expression domain. These two lines may prove to be valuable tools for further studies of the function of this protein in development and homeostasis of vertebrate organisms. Furthermore, we have cloned and sequenced the genes for CRABP-I from two other species, the chicken and the puffer fish, as is described in Chapter VI. Comparison of the coding sequences of CRABP-I from these species with those already known from other species, including mouse and man further substantiated the argument that the protein is extremely well conserved through evolution, and thus should have an important, albeit unknown, function. The promoter regions of chicken and puffer fish CRABP-I were sequenced with the aim of identifying conserved regulatory regions. This approach had proven to be successful in the identification of the regulatory regions of some of the Hox genes, but in the case of CRABP-I no conserved elements appeared to be present in the upstream region of the gene. Functional conservation of the promoter regions was tested by assessing their ability to direct the expression of a reporter gene in specific tissues in transgenic mice, in comparison with the endogenous expression pattern of mouse CRABP-I. The outcome of that study is also described in Chapter VI. Lastly, in Chapter VII, the results of the various experiments are further discussed and directions for future CRABP-I research efforts are given.

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CHAPTER II

An immunohistochemical analysis of the expression of retinoid binding proteins in the developing mouse heart.

An immunohistochemical study of the expression of retinoid binding proteins in the embryonic mouse heart.

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Abstract.

Retinoic acid (RA) is a potent teratogen during embryonic development, both in humans and in experimental animals. At physiological concentrations RA is thought to act as a morphogen. Treatment with excess RA during development results in a number of congenital malformations. One of the organs affected by this treatment is the heart. A spectrum of heart malformations is recognized, mainly affecting the outflow tract. The Cellular Retinoic Acid Binding Proteins type I and II (CRABP-I and II) and Cellular Retinol Binding Protein type I (CRBP-I) are intracellular binding proteins with a high affinity for RA or retinol respectively. In this study we have investigated the expression patterns of these retinoid binding proteins in the developing mouse heart between day 10.5 and day 15.5 of gestation by immunohistochemistry. No CRABP-I staining was detected in the heart during this period of development, whereas CRABP-II is expressed in all tissues of the heart, with the highest levels of expression in the endocardium and the trabeculated myocardium. The expression of CRBP-I colocalises with that of CRABP-II, albeit at a lower overall level. The levels of expression in the endocardial cushion tissue are low for each of these proteins. In a 12.5 day embryo of the ICR mouse strain the expression level of the proteins in the cushion tissue of both the atrioventricular canal and the outflow tract appears elevated, which may explain the remarkable specificity of this strain for a transposition of the great arteries (TGA) upon treatment with excess RA.

Introduction

Retinoic acid (RA), a naturally occurring metabolite of vitamin A, is known to have profound effects on the development of vertebrate embryos. In humans, RA has long been recognised as a potent teratogen, causing heart defects, craniofacial malformations, and central nervous system abnormalities (Lammer, 1985). A similar pattern of birth defects is observed in experimental animals (Kochar, 1967). Administration of RA to developing chicken embryos results in a continuous spectrum of cardiac malformations (Double Outlet Right Ventricle, Ventricular Septum Defect), caused by disturbance of the looping process of the heart tube and by malalignment of the outflow tract septum (Bouman, 1995; Broekhuizen, 1995), a process which is influenced by cells coming from the cardiac neural crest (Kirby, 1995). In mouse embryos RA treatment also results in heart defects. In most mouse strains, like the Swiss/FVB and NMRI strains used in this study, a wide spectrum of heart defects is recognized. Interestingly the ICR mouse strain shows a high frequency of a transposition of the great arteries

(TGA) after RA treatment, a cardiovascular anomaly which is very rarely found in other mouse strains (Pexieder, 1992; Pexieder, 1995; Yasui, 1995).

The actions of RA are mediated by two classes of proteins: The RA receptors (RARs and RXRs), and the small cytoplasmic retinoid binding proteins (CRABP-I, CRABP-II and CRBP-I). The RA receptors are transcription factors that can bind to DNA as heterodimers and transactivate upon binding of RA. They comprise of two distinct subfamilies composed of three RAR genes (RARα,β,γ) and three RXR genes (RXRα,β,γ), with each gene giving rise to multiple isoforms (Lohnes, 1992; Giguère 1994; Mangelsdorf 1994). The role of these retinoid receptors in murine development has been explored by systematic gene targeting of each member of the receptor family. Mice in which certain combinations of specific RA receptor genes have been knocked-out by gene targeting show developmental defects of the heart. The RARα/RARγ and RARα1/RARß double knock-out mice show malformations of the aortic arches and the outflow tract of the heart. The RXRα deficient mouse shows an embryonic lethal phenotype as a result of defective development of the ventricular myocardium (Kastner, 1994; Mendelsohn, 1994; Sucov, 1994; Dyson, 1995; Luo, 1996; Gruber, 1996). These findings clearly show the involvement of retinoid signaling pathway(s) in the formation of the heart.

The role of the cytoplasmic retinoid binding proteins in RA signaling is still unclear. They have been suggested to be involved in regulating the availability of RA for the nuclear receptors, either by regulating the import of RA into the nucleus (Gustafson, 1996), or by acting as a cytoplasmic buffer and influencing RA metabolism (Napoli 1996). Alternatively, it has been suggested that they could have a role in sequestering RA in the cells that require a certain concentration of RA for correct development (Lampron, 1995).

Extensive expression studies have been carried out on the retinoid binding proteins (Dollé, 1990; Ruberte, 1992) both by *in situ* hybridization and immunohistochemistry. CRABP-I, CRABP-II and CRBP-I show specific expression patterns during development, whereas CRBP-II is not expressed at embryonic stages. However, a detailed investigation of the expression patterns in the developing heart has been neglected in these studies.

We have raised effective antibodies against murine CRABP-I, CRABP-II and CRBP-I and used these antibodies for a detailed immunohistochemical investigation of the expression patterns of these proteins in the developing mouse heart from day 10.5 to day 15.5 of gestation. Their localization in specific segments of the developing heart could point to a role in heart formation. The results show specific staining patterns for CRABP-II and CRBP-I. No staining for CRABP-I was seen in the heart at any of the investigated developmental stages. An elevated level of expression of CRABP-II and CRBP-I was found in the cushion tissue of an embryo from the ICR mouse strain, in comparison to the cushion tissue of embryos of Swiss/FVB and NMRI mice, which could explain the higher sensitivity to developing TGA upon RA treatment in this strain.

Materials and methods

Production of the antibodies

Antisera against CRABP-I, CRABP-II, AND CRBP-I were obtained by immunising rabbits with synthetic peptides specific to these proteins. The peptides were chosen from presumed hydrophobic areas with minimal homology between themselves. For CRABP-I the peptide CTQTLLEGDGPKTY was chosen, for CRABP-II CEQRLLKGEGPKTS, and for CRBP-I GLEFEEDLTGIDDRKC. The peptides were coupled to keyhole limpet hemocyanin (KLH) using the heterobifunctional reagent Sulfosuccimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SulfoSMCC, Pierce Chemical Company). The conjugates were

emulsified in Freunds complete adjuvant and injected subcutaneously. Booster injections were given every second week with the antigen emulsified in Freunds incomplete adjuvant. The obtained serum needed no further purification.

Western blot analysis

The effectiveness and specificity of the antibodies was tested by Western blot analysis. Two different sets of transfection assays had to be used as it was discovered in the course of our investigation that COS7 cells contain endogenous CRBP-I. In the first assay cDNAs of the three proteins were cloned under the control of the CMV promoter in the vector pSCT-G-X556 (Rusconi, 1990). These constructs were transfected into COS7 cells by a DEAE/dextran procedure (Lake, 1991). Cells were harvested after 48 hours and cells were lysed in sample buffer for Western blotting (Laemmli, 1970). Proteins were separated on a 15% polyacrylamide gel containing 0.1% SDS, and then blotted on to 0.45 mm polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corporation). Aspecific binding was blocked by incubation of the membrane in phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA, Sigma) and 5% non-fat dried milk. The blots were incubated overnight at 4° C with the three antisera (each diluted 1:200). After washing in PBS-Tween, the strips were incubated with a 1:200 dilution of horse-radish peroxidase conjugated anti-rabbit IgG (Sigma) in PBS-Tween for 3 hours at room temperature. After washing as above, antibody binding was detected using 0.43 mg/ml diaminobenzidine (DAB) and 0.018% H_2O_2 as substratum in PBS buffer.

In the second assay we cloned the three cDNAs into the vector pEV3 (Clare Gooding, ICRF), which contains the ß globin locus control region (LCR) and promoter and gives high expression in mouse erythroid leukemia (MEL) cells after induction with dimethyl sulfoxide (DMSO) (Antoniou, 1991). The constructs were transfected by electroporation as described (Antoniou, 1991). Transfected MEL cells were induced with DMSO for four days after which cell lysates were made as for the COS7 transfections.

Source of the embryos

The embryos used in this study were obtained from pregnant females of the NMRI and Swiss/FVB strains from day 10.5 until day 15.5 post coitum, and from a pregnant ICR female at day 12.5. Embryos were collected from the uteri, washed in PBS and fixed in 35% methanol, 5% acetic acid, 35% acetone in H_2O. After dehydration in a ethanol/xylene series the embryos were embedded in paraffin. 6 μm sections were cut and applied on gelatin/$KCr(SO_4)_2$ coated glass slides. The sections were rehydrated in ethanol/xylene.

Immunohistochemical analysis

Aspecific antibody binding was blocked by pre-incubating the sections in PBS/Tween20 containing 2% NGS (normal goat serum). The sections were then preincubated with 0.3% H_2O_2 in PBS to eliminate endogenous peroxidase activity. After washing with PBS/Tween20 the sections were incubated overnight at 4°C with the primary antibodies diluted 1:100 in PBS containing 1% BSA and 0.05% Tween20. The sections were washed with PBS/Tween20 and incubated with a 1:100 dilution of peroxidase conjugated swine anti-rabbit antibody for three hours. The sections were washed with PBS/Tween20 and exposed to 0.04% diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-maleate buffer (pH 7.6) with 0.006% H_2O_2. Finally, the sections were counterstained with hematoxilin, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany).

For immunoflourescence sections were processed as follows: The slides were incubated with the primary antibodies in a 1:200 dilution overnight at 4°C. The slides were then washed three times with PBS/Tween 20 and incubated with an 1:80 dilution of Fluorescein-

isothiocyanate conjugated anti-rabbit IgG (FITC, Sigma) in PBS/Tween20/2%NGS for 6 hours at roomtemperature. The slides were washed three times with PBS/Tween20 and mounted in 1,4-diazobicyclo[2,2,2] octane (DABCO, Merck) to retard fading during microscopy. Samples were photographed using a Leica Aristoplan microscope and a Leitz Vario Orthomat.

Results

Western blot analysis

The specificity and effectiveness of the CRABP-I, CRABP-II and CRBP-I antisera was tested by western blotting (fig. 1). All three proteins appear on SDS PAGE as a band of 15 kD. When COS cells were transfected with cDNA constructs for the 3 proteins, each antiserum stained a 15 kD band in the cell lysate containing the corresponding protein. The antiserum against CRABP-I (fig. 1a) gave a slight cross-hybridisation to CRABP-II. No cross-hybridisation to CRBP-I is detected with the CRABP-I antiserum. The antiserum against CRABP-II was specific, with no cross-hybridisation to either CRABP-I or CRBP-I. Use of the CRBP-I antiserum revealed significant endogenous levels of CRBP-I in the COS cells themselves. Therefore this antiserum was tested in mouse erythroid leukemia cells (MEL C88), using the ß globin expression vector pEV3 (C. Gooding, ICRF). All 3 cDNAs were transfected into MEL C88 cells in this vector, resulting in their high level expression after induction of the MEL cells with DMSO. Cell lysates were run on a Western blot. The blot was probed with the CRBP-I antiserum (fig. 1D). A band of 15 kD is recognised in the lane with MEL cellysate transfected with CRBP-I cDNA. No bands are detected in the lanes of CRABP-I and II, showing that the CRBP-I antibody does not cross-react with either of the CRABPs.

Morphology

Between 10.5 to 15.5 day of gestation the mouse heart develops from a curved tube with a single circulation to a four chambered, completely looped and septated heart with two separate circulations. During looping the tubular heart changes into a segmented heart consisting of sinus venosus, atrium, ventricular inlet and outlet segment, which is connected to the mesenchymal walled truncus arteriosus. The segments are separated by so called transitional zones being the sinu-atrial transition, the atrioventricular canal, the primary fold and the distal outflow tract (Gittenberger-de Groot, 1995). The atrioventricular canal and the distal outflow tract are lined on the inside by endocardial cushion tissue. These two transitional zones temporarily function as valves (Moorman, 1994) In the inlet and the proximal part of the ventricular outlet segment myocardial trabeculations are recognizable. These trabeculations are connected to the myocardial compact layer. Further development of the heart shows a continued looping resulting in a wedged position of the arterial pole between both atria at day 16 (Vuillemin, 1989). During this looping process the septation in the heart proceeds. The cushion tissue of the distal outlet fuses and becomes myocardialised resulting in the subpulmonary infundibulum or outlet septum. Fusion of the atrioventricular cushion tissue results in separation of a tricuspid and mitral orifice. The primary fold, which develops between the ventricular inlet and outlet segment forms the main component of the interventricular septum. In the completely septated heart (E14.5) the inlet segment forms the larger part of the left ventricle and the outlet segment primarily forms the right ventricle. The endocardial cushion tissue of the atrioventricular canal and the outflow tract ridges seal to complete septation. The heart wall is composed of different tissues: myocardium, endocardium, epicardium and cushion tissue. The epicardium is not present yet in a 10.5 day embryo, but it starts to form shortly thereafter.

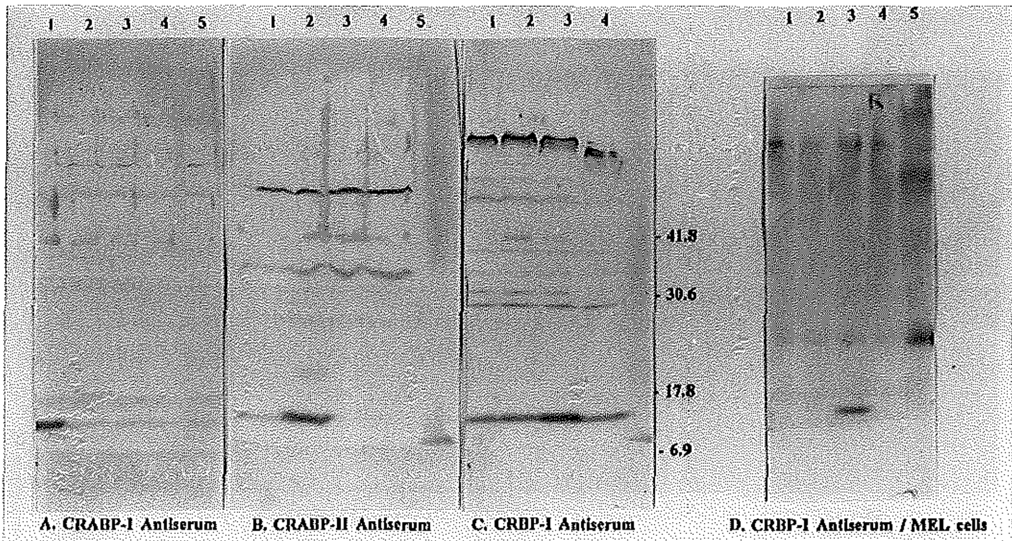

Fig. 1: Western blot analysis of polyclonal antibodies against CRABP-I, CRABP-II and CRBP-I. Cell lysates of COS7 and MEL C88 cells transfected with cDNA constructs of the three proteins were used to test the specificity of the antibodies. (A) Blot incubated with CRABP-I antiserum. Cos cells transfected with cDNA constructs of: Lane 1: CRABP-I, lane 2: CRABP-II, Lane 3: CRBP-I, Lane 4: CMV-G-X556 vector, Lane 5: untransfected COS cells. (B) Blot incubated with CRABP-II antiserum. COS cells transfected with cDNA constructs of: Lane 1: CRABP-I, Lane 2: CRABP-II, Lane 3: CRBP-I, Lane 4: CMV-G-X556 vector, Lane 5: untransfected COS cells. (C) Blot incubated with CRBP-I antiserum. COS cells transfected with cDNA constructs of: Lane 1: CRABP-I, Lane 2: CRABP-II, Lane 3: CRBP-I, Lane 4: untransfected COS cells. (D) Blot of MEL cells transfected with the three cDNAs in pEV3 and incubated with the CRBP-I antiserum. Lane 1: CRABP-I, Lane 2: CRABP-II, Lane 3: CRBP-I, Lane 4: pEV3 vector, Lane 5: untransfected MEL cells.

Immunohistochemistry

We have studied the expression pattern of CRABP-I, CRABP-II and CRBP-I in the hearts of mouse embryos between day 10.5 and 15.5 of gestation with respect to the different tissues and segments of the heart. The study was done in mice of the NMRI and Swiss/FVB strains. Additionally we have examined a 12.5 day embryo from the ICR strain. In all the stages examined CRABP-II and CRBP-I show an identical expression pattern. In all cases staining with the CRABP-II antibody was stronger than staining with the CRBP-I antibody (fig. 2). We could not detect staining with the CRABP-I antibody above the background level in the developing heart at any of the examined stages, while it was clearly detected in other parts of the embryo (e.g. central nervous system). The only site of CRABP-I staining found was in the tissue surrounding the ventriculoarterial junction at day 15.5 (Not shown). CRABP-II and CRBP-I were seen throughout the heart, but the level of staining differed between the various tissues in the heart. Staining in the compact myocardium was less intense compared to the intense staining that was seen in the trabeculae in the ventricles and in the endocardial lining of the ventricles (fig. 2). The staining in the trabeculae was remarkable in that it appeared most intense in the center of a trabecula. This was in contrast to the cells of the compact myocardium, i.e. the outer layer of the ventricular parts of the heart, where the staining appeared strongest along the cell membranes. The epicardium, from the start of its formation at around day 11, was stained at a level comparable to the level of staining found in the endocardium.

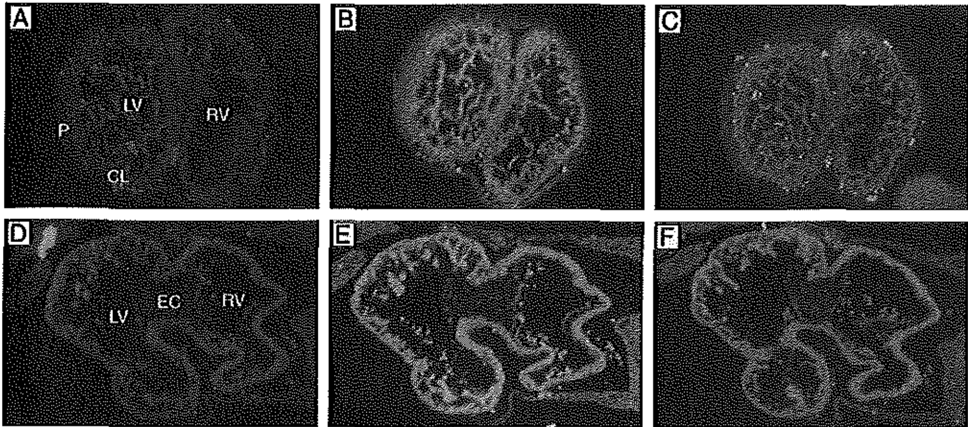

Fig. 2: Sections of day 10.5 Swiss/FVB hearts showing the left and right ventricular myocardium (A-C), and of day 11.5 hearts showing the atrioventricular canal (D-F). Immunofluorescent staining is shown with antibodies against (A,D) CRABP-I, (B,E) CRABP-II and (C,F) CRBP-I. CRABP-I staining is not found in the heart, whereas CRABP-II and CRBP-I staining is present in all tissues except in the pericardium. The relatively low expression levels in the endocardial cushion tissue are clearly visible. P: pericardium, RV: right ventricle with the trabeculated myocardium, LV: left ventricular chamber, CL: compact layer of the ventricular myocardium, EC: Endocardial cushion tissue of the atrioventricular canal.

The pericardium was negative for all three of the investigated retinoid binding proteins. The most prominent feature in the expression patterns of the retinoid binding proteins is the low level of expression in the endocardial cushion tissue, both of the atrioventricular canal and the outflow tract. Interestingly the level of staining in the endocardial cushions of the ICR strain embryo was by comparison higher, while the level of staining in the other tissues of the heart was equal to that in the NMRI and Swiss/FVB embryos (fig. 3).

At later stages (E13.5 and onwards) the expression levels of both CRABP-II and CRBP-I start to decrease. After day 15.5 no significant levels of expression are found, except for CRABP-I staining in the tissue surrounding the ventriculoarterial junction at this stage (not shown).

Discussion

Many studies have shown that correct development of the heart is influenced by retinoic acid. Recent studies on the retinoid receptors have provided a molecular basis for the effects of RA on heart formation. For example when the gene for RXRα is knocked out by gene targetting this results in embryonic lethality between E13.5 and E16.5, due to hypoplastic development of the ventricular chambers of the heart. In these animals, aberrant persistent expression of the atrial isoform of myosin light chain is seen in the ventricles. This suggests that RA provides a signal, mediated by RXRα, that is required for correct development of the ventricular myocardium. It was suggested that this signal directs the progression of the ventricular region

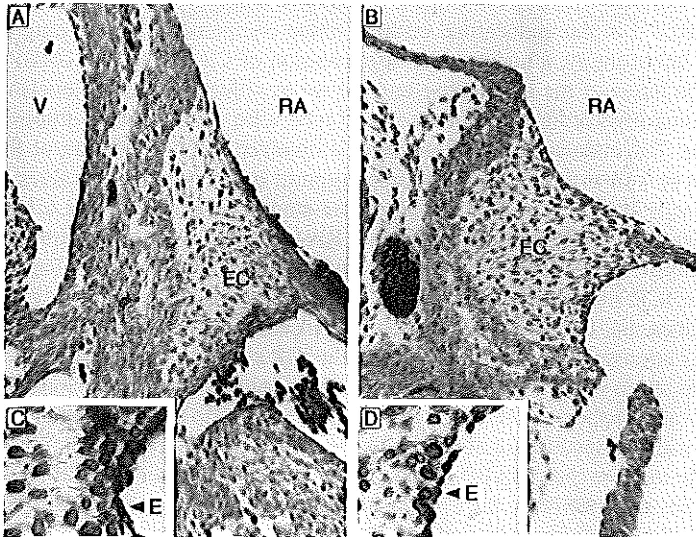

Fig. 3: Transverse sections of day 12.5 hearts at the level of the atrioventricular cushion tissue. (A) CRABP-II staining in a NMRI embryo. (B) CRABP-II staining in an ICR embryo. The highest level of staining is found in the endocardial lining (see C, D) and in the epicardial covering. A slightly higher level of CRABP-II staining was observed in the endocardial cushion tissue of the ICR embryo compared to the NMRI embryo (the black and white photograph did not allow a better visualisation of this slight difference in staining). EC: Endocardial cushion tissue, E: Endocardium, V: Ventricle, RA, Right Atrium.

from its early atrial like form to the thick walled adult ventricle (Dyson, 1995). Double knock-out mice for certain RA receptors, notably RARα/RARγ, RARα1/RARß and RARα/RARß2, also show incorrect development of the heart. The α/γ mutant suffers from myocardial deficiency, as well as outflow tract abnormalities, whereas in the a/ß2 mutant and in the a1/ß the problem seems to be restricted to outflow tract abnormalities (Mendelsohn, 1994; Luo, 1996). RA thus seems to exert its effects on heart formation via two distinct pathways using different subsets of the retinoid receptors, one being a direct effect on myocardium formation and the other affecting the neural crest which is largely responsible for the formation of the outflow tract of the heart (Gittenberger-de Groot, 1995; Noden, 1995).

The role of the retinoid binding proteins in the RA signaling pathways is still enigmatic. The question arises whether they could be intermediaries in the effects of RA on heart development? As a first step to confront this question we have looked at the distribution of these binding proteins in the heart, which could give a clue to a possible involvement. The three investigated proteins showed expression patterns that were consistent between the investigated Swiss/FVB and NMRI strains. CRABP-I is not detectable above background level in any of the tissues of the heart, except for a short exposure in the tissue surrounding the ventriculoarterial junction at day 15.5. It is therefore highly unlikely that CRABP-I has a major role in heart development. Both CRBP-I and CRABP-II are present in the developing heart leaving the question to their involvement open. However, both CRABP-II single knock-out and CRABP-I/CRABP-II double knock-out mice develop essentially normal (Fawcett, 1995; Lampron, 1995). These mice do not show heart malformations under normal RA conditions, nor a change in sensitivity to RA excess during embryogenesis. It was not reported whether the absence of CRABP-II influenced the frequency of specific heart abnormalities.

The one striking feature we found in the expression patterns is the low level of staining of both CRABP-II and CRBP-I in the endocardial cushion tissue compared to the staining in other tissues of the heart. Interestingly, a higher level of expression of CRABP-II and CRBP-I was detected in the cushion tissue of the ICR mouse embryo we examined, while the levels of expression in the surrounding myocardium and in the neural tube were comparable to those in the NMRI and Swiss mice. The endocardial cushion tissue is involved in the formation of the mitral and tricuspid valves as well as the aortico-pulmonary septum and the semilunar valves in the outflow tract. The genetic background of the ICR mouse strain causes it to show a high frequency of a transposition of the great arteries (TGA) after RA treatment, a heart defect that is very rarely seen in other mouse strains (Pexieder, 1992). It might be possible that the enhanced specificity for TGA of the ICR mouse is due to the elevated expression level of the retinoid binding proteins in its endocardial cushion tissue. In case of RA excess, more of the available RA would be preferentially attracted to the cushion tissue in the ICR strain, to deleterious effect. Further investigation of this strain will have to be performed before any definite conclusions can be drawn, but if this observation appears to be real it would be interesting to unravel the relation of its genetic background with the specific set of malformations after RA treatment. With this in mind it may be worthwhile to cross the CRABP-II knock-out allele into mice of the ICR strain to see whether this abolishes the specificity for a TGA after RA treatment and reverts it to the normal spectrum of heart abnormalities. Alternatively the creation of transgenic mice of the NMRI or Swiss strains with overexpression of CRABP-II or CRBP-I in the endocardial cushions may be easier achieved. Analysis of these mice for the incidence of TGA after RA treatment should answer the question whether the retinoid binding proteins are responsible.

Acknowledgments

The NMRI and ICR strain mouse embryos were kindly provided by Tomas Pexieder† (Lausanne). We thank Jacky Guy for critical reading of the manuscript, and Mieke Baasten for technical assistance. This work was supported by a grant from the Netherlands Heart Foundation (NWO/NHS grant 900-516-096).

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CHAPTER III

Studies on the transcriptional regulation of CRABP-I in cell lines

Studies on the transcriptional regulation of CRABP-I in cell lines.

Summary

Retinoic acid plays an important role in the development and homeostasis of vertebrate organisms, as well as in differentiation of numerous cell types. RA exerts its effect on these processes by controlling the expression of target genes via activation of the nuclear RA receptors. For correct development and differentiation it is important that the levels of RA in the different cell types are tightly controlled. The small intracellular ligand binding protein CRABP-I has a high affinity for RA. During embryonic development CRABP-I exhibits a highly specific spatiotemporally restricted expression pattern. The sites of expression coincide with the embryonic structures that are most sensitive to aberrant levels of RA. CRABP-I is thought to be an important mediator in the tight regulation of the level of RA, that is available for the nuclear receptors in specific structures of the embryo. This role obviously requires a strict regulation of the gene for CRABP-I itself, presumably taking place at the transcriptional level. This chapter describes experiments aimed at the identification of the cis-regulatory elements involved in CRABP-I transcriptional regulation.

Introduction

The widespread biological actions of retinoids are mediated by several families of serum, cytoplasmic and nuclear proteins (Giguere 1994; Ong 1994). Some of these proteins are involved in the direct effects of retinoids on gene expression, while others are involved in transport, storage and metabolism. Nuclear receptors for retinoic acid (RARs and RXRs) function as ligand inducible trans-acting transcription factors, controlling RA-dependent gene expression by interaction with cis-acting DNA elements known as RA response elements (RAREs). In addition to the RARs and RXRs, cytoplasmic binding proteins have been characterised for several retinoids, including RA (CRABPs) and retinol (CRBPs). Two different genes exist for both the CRABPs and the CRBPs ( ), and this multiplicity of cellular binding proteins suggests that the metabolism and action of retinoids is complex and tightly regulated. This is consistent with the view that retinoids, in particular RA, play an important role in vertebrate development. Various embryonic processes have been shown to be influenced by RA, including axis formation (Sive, 1990; Means, 1995), development of the central nervous system (Durston, 1989) and pattern formation in the limb bud (Eichele, 1989). The specific expression patterns observed for both the binding proteins and the receptors during mouse and chick embryonic development and in adult tissues further supports this notion (Dolle, 1990; Lyn, 1994; Ruberte, 1991/1992). The CRABP-I gene exhibits a spatio-temporally specified expression pattern during embryogenesis which correlates well with the apparent sensitivity to RA levels of the tissues concerned (Vaessen, 1990; Perez-Castro, 1990; Leonard, 1995). This implies that CRABP-I could be an important mediator in the tight regulation of the level of RA in specific structures of the developing embryo. Such an important function obviously necessitates a strict regulation of the CRABP-I gene itself, presumably taking place at the transcriptional level. Knowledge of the regulation of the CRABP-I gene will allow specific manipulation of the level and sites of its expression during development, and may thus lead to a

better insight into the role of CRABP-I in RA dependent developmental mechanisms. We have attempted to identify the cis-acting elements that are responsible for the transcriptional regulation of CRABP-I by characterization of its promoter region via CAT assays and DNAseI hypersensitive site mapping in the highly expressing cell line MES-1 (Mummery, 1986; Vaessen, 1989).

MATERIALS AND METHODS:

Isolation of genomic cosmid clones of mouse CRABP-I.

A Mouse Liver Genomic cosmid library, based on the cosmid vector pWE15 (Stratagene), was screened using a ^{32}P-labelled probe generated from the 170 basepair TaqI fragment from mouse CRABP-I (Vaessen, 1989). Hybridisation in a solution containing dextransulphate was performed as described (Sambrook, 1990). Duplicate nitro-cellulose filters were hybridised at 65°C for 16 hours, washed twice for 20 min. in 3xSSC/0.1%SDS, and once in 1xSSC/0.1%SDS (1xSSC=150mM NaCl/15mM sodium citrate, pH 7) at 65°C, and autoradiographed at -80°C with an intensifying screen. Several positive clones were picked and grown for DNA isolation. From these the cosmid clone MLGL4, containing the complete CRABP-I gene, was selected for all further manipulations. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blotting and hybridisation were performed as described (Sambrook, 1990).

Chloramphenicol acetyltransferase assay

Mes1 (Mummery, 1986) or Tera2 (Fogh, 1978) cells 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% CO_2. The cells were seeded at a density of $1x10^6$ per 6 cm dish 16 hours prior to transfection. Each dish was transfected with 5 µg of CAT-containing plasmid, and 1 µg of RSV-LacZ, using the calcium phosphate precipitation procedure (Graham, 1973) Cells were harvested 40-48 hours after transfection. Subsequently, whole cell extracts were prepared by several freeze-thaw cycles. CAT activity and ß-galactosidase activity were determined as described (Gorman, 1982).

Mapping of DNAseI hypersensitive sites

Cultured MES1 or MES68 cells were washed twice with ice cold PBS. Cells were harvested and resuspended in 800 µl of cold buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF) by gently pipetting up and down with a yellow tip. Cells were allowed to swell on ice for 15 minutes, after which 50 µl of a 10% solution of Nonidet NP-40 (Fluka) was added. The tube was vortexed vigorously for 15 seconds, and the disruption monitored by examining a drop of the suspension under the microscope. Nuclei were collected by centrifugation for 30 seconds in a microcentrifuge at 4°C. Nuclei were resuspended in cold buffer B (15 mM Tris.HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA and 5% glycerol, supplemented with 1 mM DTT, 0.15 mM spermine and 0.5 mM spermidine just before use) at a concentration of $1x10^8$ nuclei per ml. DNAseI treatment was carried out in a total volume of 500 µl of buffer B containing $2.5x10^7$ nuclei, 5 mM $MgCl_2$ and DNAseI varying in amount from 0 to 400 units. The reactions were incubated on ice for 25 minutes, and stopped by adding 10 µl of 0.5 M EDTA, 12.5 µl of 20% SDS and 50 µl of a 10

mg/ml Proteinase K solution, after which the mixture was further incubated overnight at 37 °C. After phenol/chloroform extraction the DNA was collected by isopropanol precipitation. The DNA was dissolved in 200 µl TE and digested with EcoR1 and XhoI, or with StuI. Restriction fragments were resolved on a 0.8% agarose gel and blotted onto nylon membrane (Hybond, Amersham). The blots were hybridised to the probes indicated in Figure 1.

RESULTS

Isolation of a cosmid clone containing the complete CRABP-I gene.

Genomic sequences of the mouse CRABP-I gene have been cloned previously (Wei, 1990; Vaessen, 1991), but in order to have larger stretches of genomic sequences from the CRABP-I locus available for further experiments, we have screened a mouse genomic cosmid library with a CRABP-I specific probe. We have isolated a number of overlapping cosmid clones. Clone MLGL4 appeared to contain the complete gene, which spans approximately 10 kb, as well as 20 kb of upstream sequences and 10 kb of sequences downstream of the fourth and last exon of the gene. This clone was used for all further manipulations. A map of this cosmid was constructed (Figure 1). The CRABP-I gene consists of four exons encoding 24, 59, 38 and 16 amino acids residues. The site for transcription initiation has been mapped previously to the G residue 93 bases upstream from the ATG translation initiation codon (Wei, 1990). The promoter region lacks TATA and CAAT-boxes but is GC-rich and contains 5 Sp1 binding sites in the immediate upstream region.

Fig. 1: (A) Genomic map of the murine CRABP-I locus. The four exons of the gene are indicated as stippled boxes I to IV. (B) Map of the cosmid Mlgl4, which was isolated from a mouse liver genomic library. The NotI restriction sites are derived from the cosmid vector. The location of the probes (HSS probes) that have been used in the mapping of hypersensitive sites are indicated by the striped rectangles (#1 to #8) below the map. B=BssHII, C=ClaI, E=EcoRI, EV=EcoRV, H=HindIII, S=SalI, Sn=SnaBI, St=StuI (only StuI sites in the area of the gene have been mapped), X=XhoI.

CAT assays with CRABP-I promoter/CAT reporter constructs.

The P19 derived cell line MES-1 (Mummery, 1986) and the human teratocarcinoma cell line Tera2 (Fogh 1978) both show high levels of expression of the CRABP-I gene, whereas another P19 derived cell line, END-2, does not express CRABP-I (Vaessen, 1989). To identify the promoter sequences that regulate the expression of the gene we cloned several fragments containing various CRABP-I upstream sequences in the vector pC1000CAT, which contains the CRABP-I minimal promoter driving the chloramphenicol acetyl transferase (CAT) gene. This vector, that was adapted from the vector pCAT-Enhancer (Promega), had been shown to be unable to drive CAT activity by itself, but to induce CAT activity in the presence of the SV40 enhancer element (Vaessen, 1991). A number of constructs were generated and tested for CAT activity in the MES-1 and Tera2 cell lines. The plasmid C3250-CAT contains 3.2 kb of CRABP-I upstream sequences, including the minimal promoter.

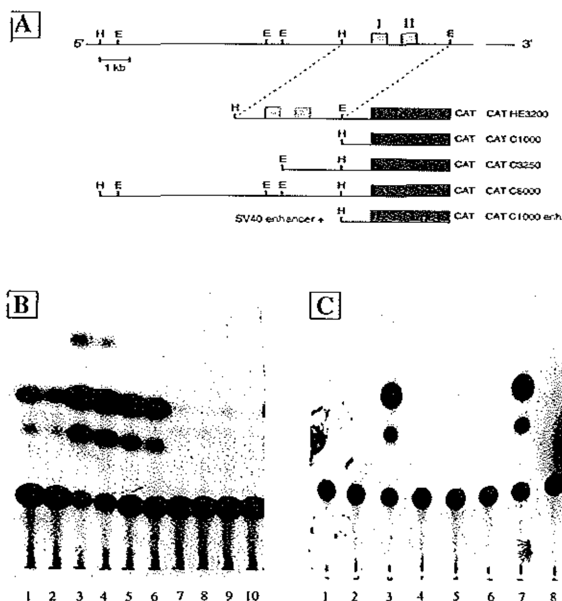

Fig. 2: CAT assays on the CRABP-I promoter region. (A) Schematic representation of the reporter plasmids used in this study. The reporter gene is the bacterial Chloramphenicol Acetyl Transferase (CAT) gene. The upstream region and the region around exons I and II of the murine CRABP-I gene is depicted at the top. Below this the various CAT reporter constructs are shown. (B and C) CAT constructs with various CRABP-I promoter fragments are unable to direct expression of the CAT gene in the cell lines (B) MES-1, END-2 and (C) Tera-2. The cell lines MES-1 and Tera-2 express CRABP-I at high level. The cell line END-2 is negative for CRABP-I and was used as a negative control. (B) Cell lysates from MES-1 cells (2,4,6,8,10) and END-2 cells (1,3,5,7,9) transfected with CAT-Enh (1,2), RSV-CAT (3,4), C1000CAT-Enh (5,6), CATC3250 (7,8) and CATC8000 (9,10). (C) Cell lysates from Tera-2 cells transfected with CATC19H (1), CATC1000 (2), CATC1000-Enh (3), CATC3250 (4), CATC8000 (5), CATHH3200 (6), RSV-CAT (7), CAT-Enh (8).

The plasmid C8000-CAT contains a further 5 kb of upstream sequences, creating a plasmid containing over 8 kb of upstream sequences. The plasmid HE3200-CAT contains the HindIII-EcoRI fragment of 3200 bp that contains 1000 bp upstream and 2200 bp downstream of the start site of the gene cloned in front of C1000-CAT. pRSV-CAT was included in the experiments as a positive control. The Rous Sarcoma Virus (RSV) long terminal repeat is known to direct accumulation of high levels of functional mRNA in many eukaryotic cell types (Gorman, 1982). pRSV-LacZ was included in each transfection to check that equal tranfection efficiencies were achieved with each of the constructs. The results of one of these CAT assay experiments is shown in Figure 2. CAT activity is clearly found in extracts of pRSV-CAT and C1000-CAT-Enhancer transfected cells. None of the other extracts contains a significant level of CAT activity. This absence of CAT activity would seem to imply that the regulatory elements that are important for the expression of the CRABP-I gene are not located within the region from -8000 to +2200 relative to the transcriptional start site of the gene. Alternatively it is possible that the chromatin structure of the CRABP-I locus is important for the activity of the enhancer elements, and in the transient transfection assays with the CAT reporter constructs that factor is not taken into account.

DNAse I hypersensitive site mapping of the CRABP-I promoter region.

The chromatin structure of promoter and enhancer regions of actively transcribed genes is often hypersensitive to DNAse I endonuclease, which reflects the accessibility of the DNA *in vivo* for interaction with proteins, like transcription factors and nucleases (Gross, 1988). As an alternative way to identify regulatory elements for the CRABP-I gene we have tried to map DNAse I hypersensitive sites in the CRABP-I locus in MES-1 cells. MES-68 cells, which do not express CRABP-I, were used as a negative control. Nuclei of these cells were incubated with various amounts of DNAseI for a short period of time. DNA was extracted, digested with restriction enzymes and separated on agarose gels. Southern blots of these gels were hybridised with various probe fragments covering the CRABP-I locus. The position of the probe fragments is indicated in Figure 1. Using probe #1 a strong hypersensitive site can be detected in MES-1 cells, which maps to the transcriptional start site of the gene. This hypersensitive site is absent in the MES68 cell line (Figure 3). Probe #1 detects an EcoRI/XhoI fragment of 2250 bp, and an EcoRI fragment of 5500 bp. Due to a heterozygous XhoI polymorphism present in the DNA of MES-1 and MES68 cells both of these bands show up as parent bands on the DNAseI hypersensitive site mapping blots with EcoRI/XhoI digested DNA. A new band appears with increasing DNAseI concentration in the MES-1 DNA samples, which has an estimated size of 3250 bp. The hypersensitive site responsible for the appearance of this band maps to the transcriptional start site of the gene. This is confirmed on the StuI blot hybridised with probe #2 where a smaller band of 7000 bp appears below the parent band of 7800 bp (Figure 3). No other DNAseI hypersensitive sites were found with any of the other six probes. Using the same blots a number of hypersensitive sites were identified in the Oct6 locus, both in the MES-1 and MES-68 cells (Mandemakers, personal communication).

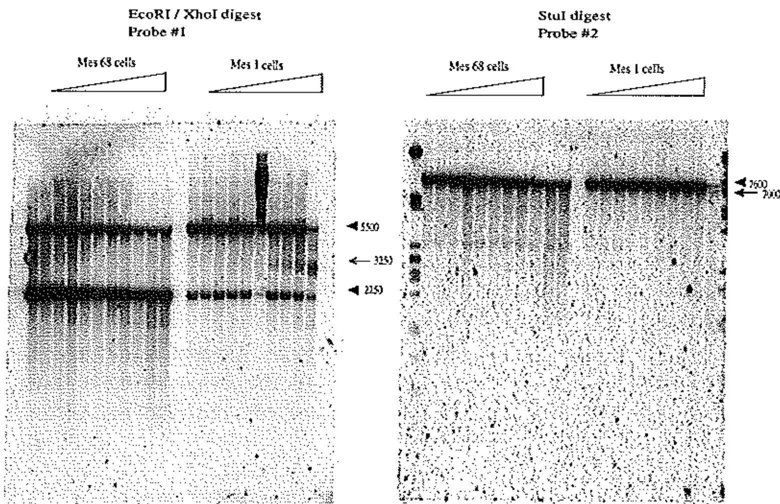

Fig. 3: DNAse I hypersensitive site assays of the mouse CRABP-I gene on intact nuclei from the CRABP-I positive cell line MES-1, and the CRABP-I negative cell line MES-68. The position of the probes used for this study is indicated in Fig. 1. The DNAse I fade out blots were made by Southern blotting of EcoRI/XhoI or StuI digested DNA isolated from DNAseI treated nuclei, and probed with probe #1 or #2. The blots shown here show the only significant hypersensitive site found, which is located at the transcriptional start site. Fragment sizes are indicated on the right.

Discussion

We have isolated a number of clones containing CRABP-I from a mouse genomic cosmid library and used one of these to compose a detailed map of the mouse CRABP-I locus. This map is in agreement with previous data (Wei, 1990; Vaessen, 1991). The promoter region of the mouse CRABP-I gene lacks a TATA-box, but is very GC rich. Five copies of the Sp1 binding site sequence GGGCGG or CCGCCC (Kadonaga, 1986) are present immediately upstream of the transcriptional start site of the gene. It had been shown previously that a 1 kb fragment containing these immediate upstream promoter sequences cloned in a CAT reporter gene construct and transfected into MES-1 cells, could not produce detectable levels of CAT activity. When an additional enhancer element, the SV40 enhancer, was inserted into the CAT reporter gene construct, the resulting construct exhibited clear stimulation of CAT expression. These results indicated that the 1 kb genomic fragment containing the CRABP-I promoter region contains minimal promoter activity, but that this minimal promoter is by itself insufficient to drive expression of the CAT gene. It is apparent that additional enhancer elements are required. To identify and locate these putative enhancer elements we have constructed an additional set of CAT reporter gene constructs, covering the region from -8800 to +2250 bp relative to the transcriptional start site of the gene. CAT activity of these constructs

was assayed in the highly CRABP expressing cell lines MES-1 and NTera2. None of the constructs appeared to exhibit CAT activity, whereas the positive controls pRSV-CAT and pC1000CAT-Enh clearly showed expression of the CAT gene. These results would seem to indicate that the putative CRABP-I enhancer elements are located outside of the investigated region. But the absence of stimulatory effects on the transiently transfected CAT reporter gene constructs may also reflect the importance of the local chromatin structure in the regulation of the CRABP-I gene.

As an alternative means to identify and locate the putative enhancer elements we have tried to map DNAseI hypersensitive sites in the CRABP-I locus. DNAse I hypersensitive sites are often found at the promoter and enhancer regions of actively transcribed genes, because a more open chromatin structure is present at these sites in the DNA *in vivo*, due to, or required for, the binding of transcription factors (Gross, 1988). Only one significantly strong hypersensitive site could be detected in MES-1 cells. This hypersensitive site maps to the transcriptional start site of the gene. In the MES68 cell line, which does not express CRABP-I, this site is not present. Thus the presence of the DNAseI hypersensitive site at the transcriptional start site of the gene correlates well with its transcriptional status, at least in these two cell lines. The same blots were used to identify a number of hypersensitive sites in the Oct6 locus (Mandemakers, personal communication), indicating that the blots were fit for the detection of hypersensitive sites. However, we were unable to detect other DNAseI hypersensitive sites in the CRABP-I locus.

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CHAPTER IV

REGULATION OF THE CRABP-I GENE DURING MOUSE EMBRYOGENESIS

Regulation of the CRABP-I gene during mouse embryogenesis.

Part of this chapter has been published in:

Dirk A. Kleinjan[⊜], Sylvia Dekker, Marie-Josée Vaessen and Frank Grosveld[*]. Regulation of the CRABP-I gene during mouse embryogenesis. (1997); Mechanisms of Development **67**; 157-169.

Summary

The Cellular Retinoic Acid Binding Protein type I (CRABP-I) shows a highly specific expression pattern during mouse embryonic development. The tissues that express CRABP-I, i.e. the central nervous system (CNS), neural crest, branchial arches, limb bud and frontonasal mass, coincide with those that are most sensitive to unphysiological retinoic acid (RA) concentrations. We have investigated the transcriptional elements that are responsible for the spatiotemporal specific regulation of CRABP-I expression in the mouse embryo. To identify the regulatory regions of the gene we have inserted a Myc epitope tag into the coding sequence of the gene and used this tag as a reporter to study the expression patterns of the transgenes. This strategy was chosen so that we altered the endogenous situation of the gene as little as possible in our transgenic constructs, while at the same time this would allow the use of the same constructs to generate mice with overexpression of CRABP-I under control of its own regulatory sequences. We show here that a 16 kb fragment surrounding the gene harbours all the elements needed for the correct spatiotemporal expression pattern. Upon further dissection of this fragment we found that expression in the CNS is driven by elements in the upstream region of the gene, while expression in mesenchymal and neural crest tissue is regulated via element(s) located downstream of exon II of the gene. Thus, distinct enhancers are involved in the transcriptional regulation of the CRABP-I gene in different tissues of the developing mouse embryo. Two of the transgenic lines generated in this study show spatiotemporally specific overexpression of the tagged protein. We show that the presence of the epitope-tag sequences does not interfere with the RA binding capacity of the tagged CRABP. However, mice from both of these lines are apparently normal under normal laboratory conditions.

Key words: CRABP-I, transcriptional regulation, transgenic mice.

Introduction

Retinoic acid (RA), a naturally occuring metabolite of vitamin A, plays a vital role in normal physiology of vertebrates. Maintainance of physiological levels of RA is important for correct embryonic development since both excess and deficiency of RA result in a spectrum of congenital malformations (Wilson, 1953; Kochhar, 1967). Under normal conditions RA is believed to be involved in the anterior-posterior patterning of the embryo, including the central nervous system (CNS) (Means, 1995).

The profound effects of RA on biological systems are mediated through two classes of proteins: the retinoic acid receptors (RARs and RXRs) and the cellular retinoic acid binding proteins (CRABPs). The RARs/RXRs are ligand inducible nuclear receptors, belonging to the steroid/thyroid hormone receptor superfamily (Mangelsdorf, 1995; Chambon, 1996). They regulate gene expression through binding as heterodimers to specific DNA sequences, RA response elements (RAREs), contained in the regulatory regions of responsive genes. Within each receptor family (RAR or RXR) three different genes have been identified, each giving rise to multiple isoforms (Leid, 1992). The CRABPs are small intracellular proteins that bind RA with high affinity. Two highly homologous but different CRABP genes (CRABP-I and CRABP-II) have been cloned in a number of species. They appear to be highly conserved through vertebrate evolution. Each CRABP specifically binds RA, with a higher affinity than the RARs. The RA binding affinity of CRABP-I is four fold higher than that of CRABP-II (Norris, 1994; Napoli, 1995). They are likely to have a role in regulating the availability of RA to the nuclear receptors, but their exact function remains to be demonstrated.

CRABP-I shows a spatiotemporally specific expression pattern during embryonic development, with expression found in the central nervous system (CNS), the neural crest, the dorsal root ganglia, the limb bud and the frontonasal mass (Ruberte, 1991; Ruberte, 1992; Lyn, 1994; Horton, 1995). In the limb bud a graded distribution of CRABP has been found along the proximo/distal axis. Some authors have also observed a gradient along the anterior/posterior (A/P) axis of the limb (Maden, 1988; Perez-Castro, 1989), but the presence of an A/P gradient was not found by others (Dolle, 1989; Ruberte, 1992). In the CNS the protein is expressed in the outer layer of the midbrain roof, in the hindbrain and in the mantle layer of the spinal cord. Interestingly these sites of expression coincide with the structures that are most sensitive to RA excess (Vaessen, 1990). Both CRABP-I and CRABP-I/CRABP-II deficient mice have been generated (de Bruijn, 1994; Gorry, 1994; Lampron, 1995), but as yet no abnormal phenotype has been observed in these mice. It has been suggested that the function of CRABP-I may only become apparent under conditions of RA deficiency, when it could preferentially sequester RA in those tissues that are critically dependent on the availability of the ligand (Lampron, 1995). Overexpression of the protein in F9 cells (Boylan, 1991) and ectopic expression of the protein under control of a heterologous promoter in transgenic mice (Wei, 1991; Perez-Castro, 1993) have both been shown to interfere with normal cellular differentiation. Overexpression of CRABP (xCRABP) in *Xenopus* was found to cause anteroposterior defects in developing embryos (Dekker, 1994). Thus, while the knock-out of CRABP-I did not reveal its function, overexpression of the protein may give some indication of its role in embryonic development. Knowledge of the regulatory elements would open the possibility to overexpress CRABP-I within its endogenous expression domain, or in specific subdomains thereof, and provides a means to characterise the transcription factors that play a role in early neural and neural crest development.

We show here that a 16 kb construct, GCTag, is able to regenerate the complete expression pattern of endogenous CRABP-I. Deletional analysis of this construct revealed that the expression pattern of the transgenic CRABP can be split into two domains: a neural and a mesenchymal/neural crest component. Expression in the mesenchymal/neural crest domain is driven by element(s) located downstream of exon II of the gene. Furthermore we have generated two transgenic lines which overexpress CRABP within its own expression domain. Mice from both lines appear normal under normal laboratory conditions.

Materials and Methods

Constructs

A 5500bp genomic EcoRI fragment containing exons I and II of the murine CRABP-I gene was subcloned, and the C at position +4 of the coding sequence was changed to a G by site-directed mutagenesis, thus creating an NcoI site at the translational start site. A 30 bp sequence coding for a 10 amino acid c-myc derived tag (Evan, 1985) was cloned into this site, to create pDJTag. Addition of the 3' end of the gene to pDJTag resulted in pGCTag (see Fig.1). The microinjection fragments GCTag and XHTag were isolated from this plasmid. The cosmid M4Tag was created by adding 5' and 3' flanking regions back to GCTag. pECTag was constructed by linking the cDNA sequences for exons II,III and IV in frame to exon II of pDJTag. The microinjection fragments ECTag, SCTag and XCTag were all derived from this plasmid.

Transgenic mice production and processing of the embryos

Microinjection was performed according to standard procedures (Hogan, 1994). Transgenic mice and embryos were identified by Southern blot analysis. Embryos were collected at midgestational stages. For detection of Myc-tagged CRABP-I the embryos were washed in PBS (Phosphate Buffered Saline) and fixed for 1 hour in 35% methanol, 35% acetone, 5% acetic acid. For staining with X-gal, embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM ethylene glycol-bis(beta-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.02% Nonidet P-40 (NP-40).

Analysis of the embryos

After fixation, transgenic embryos containing the Myc-tag were dehydrated, embedded in paraffin and sectioned. After rehydration through ethanol/xylene, aspecific binding was blocked by pre-incubating the sections in PBS/Tween20 containing 2% NGS (normal goat serum). To eliminate endogenous peroxidase activity the sections were preincubated with 0.3% H_2O_2 in PBS. The sections were incubated overnight at 4°C with primary antibodies against CRABP-I or the Myc epitope in a 1:100 dilution in PBS containing 1% BSA and 0.05% Tween20. The sections that were incubated with the CRABP-I antibody were then incubated with a 1:100 dilution of peroxidase conjugated swine anti-rabbit antibody for three hours. Next the sections were exposed to 0.04% diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-maleate buffer (pH 7.6) with 0.006% H_2O_2. The Myc-epitope antibody incubated sections were incubated with an alkaline phosphatase conjugated goat anti-mouse antibody and then exposed to nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). Finally, the stained sections were dehydrated and mounted.

Embryos transgenic for LacZ constructs were stained for several hours or overnight at 37°C in the dark in a solution containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, 0.01% sodiumdeoxycholate, 0.02% NP40 and 0.1% 5-bromo-4-chloro-3-indolyl-ß-D-galacto-pyranoside (Xgal).

Western blot analysis

To test whether our CRABP-I antibody and the monoclonal antibody against the Myc epitope tag would effectively recognise the CRABP-Tag protein we performed a Western blot. CRABP-I and CRABP-Tag cDNAs were cloned under the control of the CMV promoter in the vector pSCT-G-X556 (Rusconi 1990), resulting in the constructs CMV-CRABP-I and CMV-

CRABP-Tag. These constructs, as well as the parent vector pSCT-G-X556, were transfected into COS7 cells by a DEAE/dextran procedure (Sambrook, 1989). Cells were harvested after 48 hours and lysed in sample buffer for Western blotting. Proteins were separated on a 15% polyacrylamide gel containing 0.1% SDS, and then blotted on to 0.45 μm polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corporation). Aspecific binding was blocked by incubation of the membrane in phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA, Sigma) and 5% non-fat dried milk. They were incubated overnight at 4° C with the three antisera (each diluted 1:200 for each of the three antisera). After washing in PBS-Tween, the strips were incubated with a 1:200 dilution of horse-radish peroxidase conjugated anti-rabbit IgG (Sigma) in PBS-Tween for 3 hours at roomtemperature. After washing as above, antibody binding was detected using 0.43 mg/ml diaminobenzidine (DAB) and 0.018% H_2O_2 as substratum.

RA binding assay

COS cell transfections were done with CMV-CRABP-I, CMV-CRABP-Tag and the parent vector pSCT-G-X556 as described above. Cells were harvested after 48 hours and whole cell extracts were made from transfected cells as well as untransfected COS cells, END2 cells and MES1 cells (Mummery, 1987). 20 pmol of [11,12-^{3}H]-retinoic acid (NEN products) was added to each protein sample in a total volume of 100 μl and incubated in the dark on ice for 6 hours. For competition, 10 fold or 100 fold excess of unlabeled retinoic acid (Sigma) was added to the sample. To remove unbound [11,12-^{3}H] RA, each sample was mixed with 400 μl of dextran-charcoal suspension, consisting of 0.8& charcoal, 0.08% dextran T70, 0.25 M sucrose, 1 mM EDTA, 10 mM Tris.HCl (pH 7.5) for 30 minutes, and then centrifuged at 5000 rpm for 20 minutes at 4°C. After centrifugation, a sample was taken from the supernatant and the amount of radioactivity was determined in a scintillation counter.

RNA isolation and S1 analysis

Isolation of RNA from the transgenic mice used in this study was done as follows. Pregnant females were sacrificed at 11.5 days of gestation and embryos were collected. Transgenic embryos and non-transgenic littermates were identified by Southern blotting. Copy numbers were determined by comparison of the endogenous CRABP-I signal and the signal of the transgenic CRABP-Tag. Whole embryos were homogenised in 2 ml of 6 M Urea, 3 M LiCl for approximately 40 seconds and sonicated for 1 minute. RNA was allowed to precipitate overnight at 4°C and collected by centrifugation at 10g for 30 minutes at 4°C. Pellets were washed once in the same solution, dissolved in 10mM Tris.HCl (pH 7.5), 0,5% SDS, phenol/chloroform extracted and ethanol precipitated.

CRABP-I and CRABP-Tag RNAs were analysed by S1 nuclease protection assay. Probes were generated from the N-terminal region of the gene in such a way that the protected fragments derived from CRABP-I and CRABP-Tag RNA are distinguished by size. A probe for mouse ß-actin was included as loading control. The probes were end-labelled with T4 polynucleotide kinase. 25 ng of labelled probe was hybridised to 10 μg of total embryo RNA in a total volume of 15 μl of 40 mM Pipes (pH 6.4), 400 mM NaCl, 1 mM EDTA and 80% formamide for 16 hours at 56°C. Samples were digested for 2 hours at 25°C with 100 units of S1 nuclease (Boehringer) in 250 μl of 200 mM NaCl, 30 mM NaOAc (pH 4.5), 2 mM $ZnSO_4$. The protected DNA was phenol/chloroform extracted, ethanol precipitated and loaded on a 6% urea-polyacrylamide gel. After electrophoresis the gel was dried, and scanned on a PhosphoImager using ImageQuant software (Molecular Dynamics).

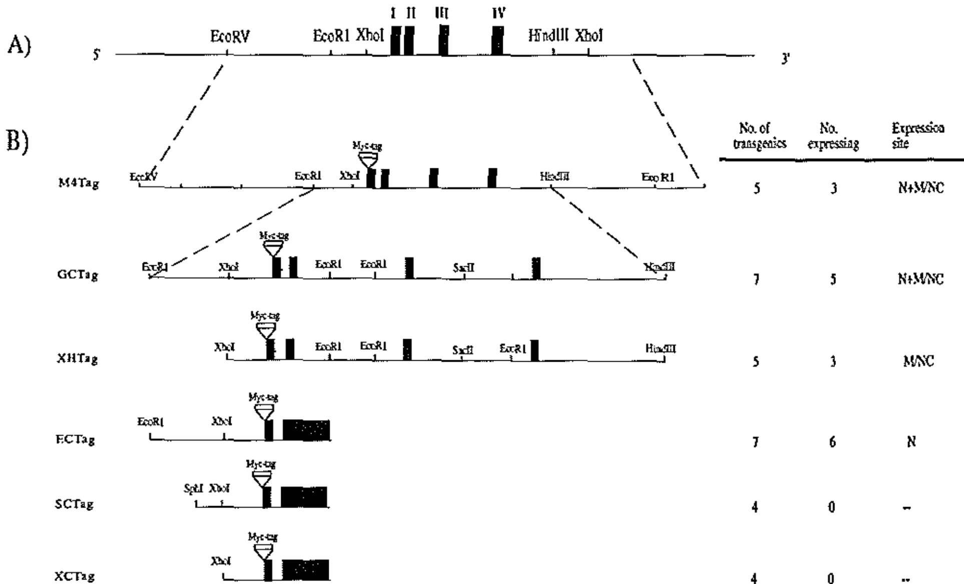


Fig. 1. A Genomic locus of murine CRABP-I. Black boxes indicate the four exons of the gene. **B** Fragments used to generate transgenic mice. All fragments contain the complete CRABP-I coding sequences, into which an epitope tag, derived from human c-Myc, has been inserted. M4Tag contains 20 kb upstream sequences and 20 kb downstream. The 16 kb fragment GCTag contains 3.2 kb upstream sequences and 13 kb downstream. XHTag has 1.1 kb upstream sequences and 13 kb downstream. ECTag contains 3.2 kb upstream sequences and is fused in exon II to the CRABP-I cDNA. SCTag and XCTag are derived from ECTag, and contain 1.7 kb and 1.0 kb of the upstream region respectively. The table on the right side indicates the expression of the different constructs in transgenic mice. The first column is the number of transgenic mice, the second column the number of mice that express the transgene, and the third column indicates the site of expression. N indicates expression in the neural subdomain of CRABP-I expressing cells. M+NC indicates expression in neural crest cells and the mesenchymal subdomain of CRABP-I expressing cells.

Results

To define the genomic sequences that direct the expression of the CRABP-I gene in the mouse embryo we have generated a number of genomic reporter constructs from the CRABP-I locus and analysed their expression patterns in transgenic founder embryos and transgenic lines at various developmental stages. The constructs used for micro-injection in this study are illustrated in Fig. 1. In order to preserve the genomic organisation of the locus as much as possible the transgenic constructs consist of fragments from the murine CRABP-I locus containing the complete CRABP-I coding region. To distinguish between expression of the transgene and the endogenous CRABP-I, the transgenic CRABP has been marked with an epitope tag derived from the human c-myc proto-oncogene (Evan , 1985). Using site-directed mutagenesis an NcoI restriction site was created at the translational start site of the CRABP-I gene, into which the Myc epitope tag was cloned. Constructs containing this tagged CRABP-I,

hereafter referred to as CRABP-Tag, were microinjected into mouse oocytes. Embryos that were identified as transgenic by Southern blotting were embedded, sectioned and stained for CRABP-I and for the Myc-tag.

A 16 kb fragment GCTag can regenerate the CRABP-I expression pattern

The cosmid construct M4Tag contains 40 kb of the CRABP-I locus, of which 20 kb are located upstream of the CRABP-I start-site. Five independent lines were obtained that were transgenic for this construct. Two out of five lines did not show expression of the transgene, probably due to integration in an area of the mouse genome that is transcriptionally silent. Injection of the 16 kb fragment GCTag, with 3.2 kb upstream sequences and 13 kb downstream sequences yielded seven independent transgenic animals. Four were bred as lines and three were isolated as founder embryos. Of the founder embryos two did not express the transgene. The expression patterns of the transgenes M4Tag and GCTag were essentially identical. The expression patterns of CRABP-Tag in the expressing lines were analysed in embryos at midgestational stages. Expression of CRABP-Tag in these embryos was found in the central nervous system, the limb buds, the mesenchyme in the mesonephric area, and in the frontonasal mass (Fig. 2A-D). In the CNS staining for CRABP-Tag is found in the outer layer of the midbrain, in the hindbrain and the mantle layer of the spinal cord. In a 10.5 day p.c. embryo staining is found in the outer layer of the hindbrain and throughout the thickness of rhombomeres 2, 4, 5 and 6, while being absent from rhombomeres 1 and 3 (Fig. 2C), as has been found for endogenous CRABP-I (Maden , 1992; Leonard , 1995). At later stages CRABP-Tag staining in the hindbrain is only found in the outer layer. Migrating neural crest cells on either side of the spinal cord, and the neural crest derived dorsal root ganglia are also positive for CRABP-Tag (Fig. 3A,B). In the limb buds of day 10.5 p.c. embryos CRABP-Tag staining is found in a graded manner with the highest levels found at the distal end (Fig. 2D). We found no consistent evidence for a graded distribution along the anteroposterior axis. At 13.5 days CRABP-Tag protein is found in the proximal interdigital region in the cells surrounding the cartilaginous condensations (not shown). In addition to this we find expression in the otic vesicle (Fig. 2B,D). CRABP-I is expressed in the mesenchyme in the branchial arches of 9.5 d.p.c. embryos, with weak expression in the first arch and slightly stronger expression in the second and third arch, and disappears around 10.5 d.p.c.. We do not normally detect CRABP-I staining with our CRABP-I antibody in 10.5 d.p.c. embryos, except after prolonged stainings. In some of the transgenic lines weak expression is seen in the branchial arches of 9.5 - 10 d.p.c. embryos, but no expression was found in 10.5 d.p.c. and older embryos. The overall pattern of expression is consistent with the endogenous expression pattern of CRABP-I. These results show that the 16 kb fragment GCTag contains all the elements required for the correct regulation of CRABP-I expression during mouse embryogenesis.

The CRABP-I expression pattern can be divided into a neural and a mesenchymal/neural crest component

In order to further localise the elements regulating CRABP-I expression we made the constructs ECTag, XHTag, SCTag and XCTag (see Fig.1). ECTag is a hybrid genomic/cDNA construct, containing 3 kb of 5' sequences, the first exon containing the Myc-tag, the first intron, and the cDNA sequences of exons 2, 3 and 4. This fragment was injected to determine the

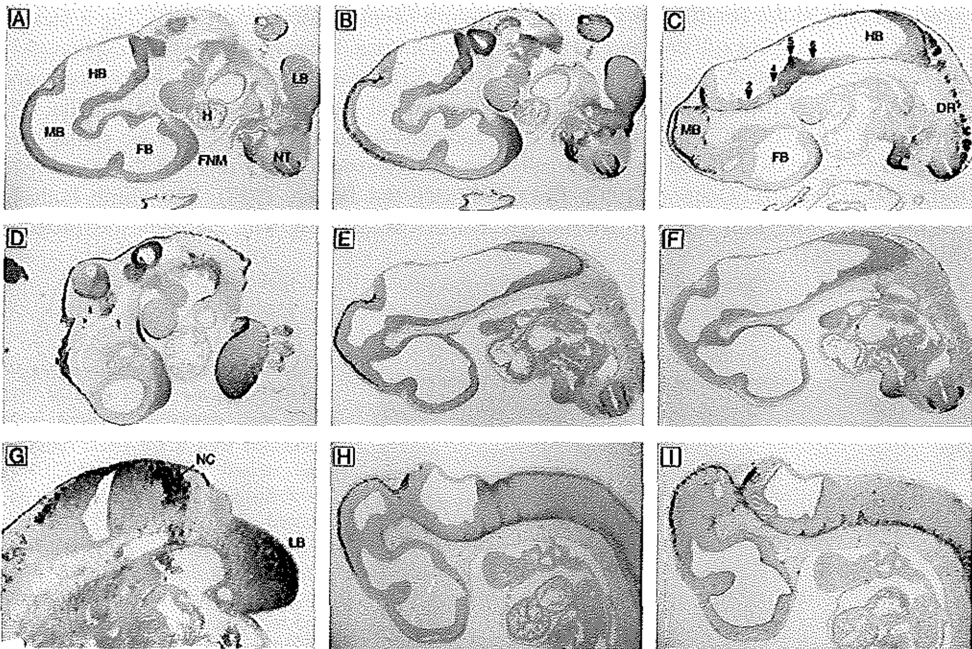


Fig. 2. Immunohistochemical analysis of GCTag, XHTag and ECTag transgenic mouse embryos at 10.5 days p.c.. Sections were incubated with an antibody against CRABP-I and stained with DAB (A,E,H), or with a monoclonal antibody against the Myc epitope tag and stained with BCIP/NBT (B,C,D,F,G,I). Sections of transgenic embryos with construct GCTag. The full CRABP-I expression pattern is reproduced by the transgene, with expression found in the midbrain, hindbrain, spinal cord, dorsal root ganglia, limb bud and frontonasal mesenchyme (A,B,C,D). Rhombomere specific staining is seen at low level in rhombomere 2 and at higher level in rhombomeres 4, 5 and 6 (C). Staining in the limb bud shows a gradient along the proximo/distal axis with highest levels of staining found at the distal ends (D,G). Sections of a transgenic embryo carrying construct XHTag. Myc-tag staining is found in mesenchymal and neural crest tissue, but not in neural tissue. The lack of transgene expression in the midbrain is evident (E,F,G). Transgene expression in an XHTag embryo shows the expression in the neural crest and the proximo/distal gradient in the limb bud (G). Sections of an embryo from an ECTag transgenic line (H,I). The transgene (I) is expressed only in neural cells that form a subset of the CRABP-I expressing cells (H). FB, forebrain; MB, midbrain; HB, hindbrain; H, heart; FNM, frontonasal mesenchyme; LB, limb bud; DR, dorsal root ganglia; NC, neural crest.

presence of regulatory sequences in the 5' region of the gene. Six out of seven ECTag lines showed expression of the transgene, but expression was found only in the midbrain, the hindbrain and the ventral aspect of the mantle layer of the spinal cord, i.e. the neural tissues that express CRABP-I (Fig. 2H,I and 3E,F). None showed expression in any of the other CRABP-I expression sites. None of 8 SCTag and XCTag lines, which contain 1.7 and 1.0 kb of upstream sequences, the tagged exon 1, intron 1, and exons 2, 3 and 4 from the cDNA expressed. Thus the minimal promoter region is insufficient to direct expression without the upstream sequences between EcoRI and XhoI, containing the putative control elements for expression in neural tissue.

The construct XHTag was designed to test whether intragenic or 3' sequences are involved in regulation of CRABP-I expression. It starts at the XhoI site 1 kb upstream of the CRABP-I gene and ends at the HindIII site 2 kb after the fourth exon. In comparison to GCTag it lacks 2 kb of 5' sequences, but still contains all the exons and introns. Four out of five lines expressed the transgene. Strikingly, the pattern of expression of XHTag is complementary to the one found with ECTag, and includes the limb bud, migrating neural crest cells, dorsal root ganglia, otic vesicle, mesenchyme in the mesonephric area, and cells in the dorsal aspect of the spinal cord (Fig. 2E,F,G and 3C,D). The latter are cells that are believed to have retained the potency to form neural crest (LeDouarin, 1982; Bronner-Fraser and Fraser, 1988). The limb buds of XHTag mice also show a proximo/distal gradient of CRABP-Tag with the highest expression distally (Fig. 2G).

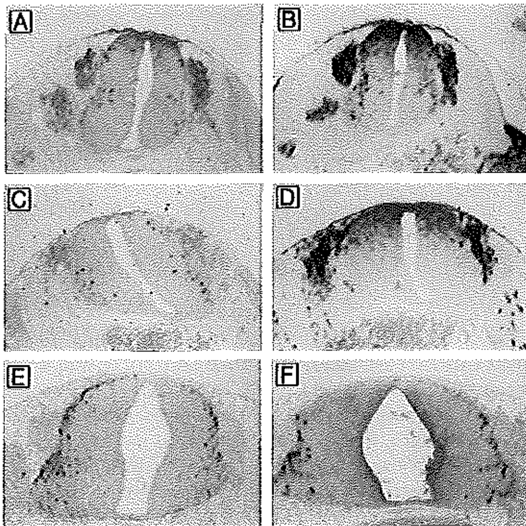


Fig. 3. Expression patterns of tagged CRABP-I in the spinal cord of 10.5 days p.c. embryos. Sections were incubated with a CRABP-I antibody and stained with DAB (A,C,E) or incubated with a Myc-tag antibody and stained with BCIP/NBT (B,D,F). GCTag transgenic embryos show expression in the mantle layer of the spinal cord, in the dorsal root ganglia and in the neural crest cells migrating on either side of the spinal cord (A,B). XHTag containing embryos express the transgene in the dorsal aspect of the spinal cord and in the migrating neural crest cells (C,D), while ECTag transgenic embryos express the transgene in the mantle layer of the spinal cord except in the dorsal most portion (E,F). DR, dorsal root ganglion, NC, neural crest cells, NT, neural tube, ML, mantle layer.

The pattern seen with GCTag can be reconstructed by overlaying the expression patterns of ECTag and XHTag. This shows that the CRABP-I gene is regulated via at least two independent enhancer regions, one region responsible for the neural component of its expression, located upstream of the gene, and one for its mesenchymal/neural crest component, located downstream from exon II.

Overexpression of CRABP-Tag in transgenic mice

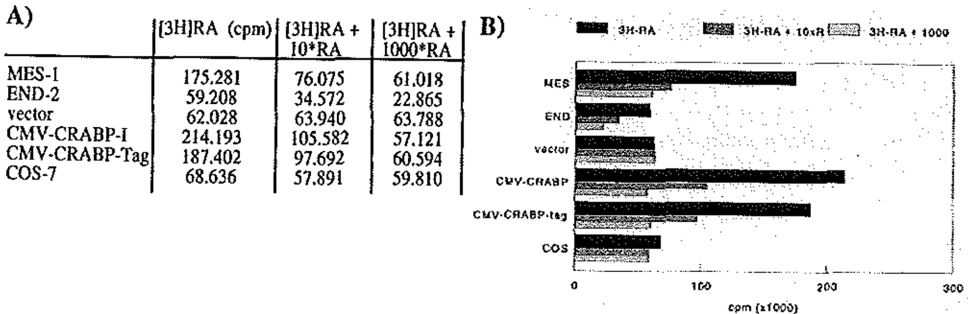
Our other objective with using the genomic CRABP-I locus containing the complete coding sequences of the gene to generate transgenic mice was the overproduction of CRABP in those mice. The sole difference between CRABP-I and CRABP-Tag is the presence of the 10

amino acid peptide sequence used as the epitope tag. We first investigated whether the presence of this epitope would interfere with CRABP-I function. We have performed an RA binding assay, and compared the RA binding ability of the tagged form of CRABP, referred to as CRABP-Tag, with the wildtype form of CRABP-I. For this purpose we have cloned the Myc epitope tag in the cDNA of CRABP-I at the same N-terminal position of the coding sequence as was used in the genomic constructs for the production of transgenic mice, resulting in a CRABP-Tag cDNA. Both the CRABP-I and CRABP-Tag cDNAs were then inserted into the expression vector pCMV-G-X556 (Rusconi, 1990), and transfected into COS-7 cells. Cell lysates were made 48 hours after transfection. Cell lysates of the CRABP-I expressing MES-1 and the non-expressing END-2 cell lines were used as controls. The expression from the CRABP-I and CRABP-Tag expression constructs was assayed by Western blot (not shown). CRABP-I runs as a 15 kD band on the blot incubated with the CRABP-I antibody, while CRABP-Tag runs as a 18 kD due to the presence of the additional amino acid residues of the Myc epitope. The RA binding abilities of the COS cell expressed CRABP-I and CRABP-Tag proteins were then compared in an *in vitro* RA binding assay (figure 4). An aliquot of the cell lysates was incubated with radioactively labeled [11,12 ^{3}H] RA for several hours in the dark, in the presence or absence of excess unlabeled RA. After removal of excess labeled RA, CRABP bound radioactivity was measured in a scintillation counter. The results presented in fig. 4 show that the RA binding capacity of CRABP-Tag is nearly identical to that of CRABP-I, indicating that the presence of the epitope tag does not interfere with the RA binding function of CRABP-I. Unlabeled RA can effectively compete with labeled [11,12 ^{3}H]RA, showing that the binding of RA is specific and indicative of the RA binding capacity of the lysates.

The complete expression pattern of CRABP-I is reconstituted in transgenic mice that express the constructs M4Tag and GCTag. To determine the level of expression of CRABP-I and CRABP-Tag in these animals we have performed S1 analyses on embryos isolated from the various transgenic lines (figure 5). Total RNA was isolated from whole embryos at 10.5 days p.c. and hybridized to the probes depicted in fig. 5A. The exon I probe is derived from a construct in which the upstream region of CRABP-I is linked to the CRABP-Tag cDNA, termed pSalcDNATag. The probe contains the N-terminal sequence of the construct and discriminates between CRABP-I transcripts and CRABP-Tag transcripts. Hybridisation of the exon I probe with CRABP-Tag gives a protected fragment of 210 basepairs, while hybridisation to the wildtype CRABP-I gives a 90 basepair protected fragment. The exon II probe is derived from the construct ECTag and generates protected fragments of equal length of 150 basepairs with both CRABP-I and CRABP-Tag mRNA. A mouse ß-actin probe was included in all samples as a control for the presence of equal amounts of RNA in the samples. The result from a typical S1 analysis is shown in fig. 5. The transgenic lines GCtag7 and M4Tag9 express CRABP-Tag at a level that is below the endogenous CRABP expression level. The same result was found for the lines GCTag5, GCTag12 and M4Tag1 (not shown). The lines GCTag3 and M4Tag4 however show a clear overexpression of CRABP-Tag. The total level of expression in both these lines is estimated at 3 to 5 times the level of expression of the endogenous CRABP-I. The presence of high levels of the transgene does not result in a downregulation of endogenous CRABP-I, as the level of CRABP-I is approximately equal in wildtype, GCTag3 and M4Tag4 embryos.

Mice from these lines have been under observation for more than 12 months. No apparent abnormal phenotype could be detected in either the GCTag3 or the M4Tag4 lines. Transgenic embryos, isolated between 9.5 days p.c. and 14.5 days p.c., do not show any apparent developmental malformations. Thus overexpression of CRABP appears not to have an adverse effect on these mice under normal laboratory conditions. Experiments in which the mice

are subjected to vitamin A deficiency or excess will have to reveal the putative function of CRABP-I in a situation of vitamin A stress.



A)

	[3H]RA (cpm)	[3H]RA + 10*RA	[3H]RA + 1000*RA
MES-1	175.281	76.075	61.018
END-2	59.208	34.572	22.865
vector	62.028	63.940	63.788
CMV-CRABP-I	214.193	105.582	57.121
CMV-CRABP-Tag	187.402	97.692	60.594
COS-7	68.636	57.891	59.810

B)

Figure 4: Retinoic acid binding assay. Cell lysates from COS-7 cells transfected with expression constructs for CRABP-I, CRABP-Tag or the parent vector, as well as untransfected COS-7, MES-1 (CRABP positive) and END-2 (CRABP negative) cells were incubated with radioactively labeled RA, in the presence or absence of excess unlabeled RA. After removal of unbound RA, the amount of radioactivity present in a fraction of the incubation mixture was counted (A). Bar diagram of the results (B).

Discussion

The CRABP-I gene shows a specific expression pattern during embryonic development. As retinoic acid is critically involved in pattern formation of vertebrate embryos, the spatiotemporally restricted expression of CRABP-I suggests it may be involved in controlling the level of RA in different tissues of the embryo. An understanding of the factors that control expression of CRABP may provide further insight into the mechanisms of RA signal transduction during embryogenesis. Furthermore, identification of the cis-acting elements would allow manipulation of CRABP-I levels or related proteins in specific tissues in the embryo. We have therefore investigated the molecular mechanisms that are involved in the regulation of CRABP expression in transgenic mice. We show here that multiple sets of enhancer elements are employed by the gene. The complete spatial and temporal expression pattern of the gene was reproduced in transgenic mice with the 40 kb cosmid M4Tag, which contains the complete CRABP-I coding region plus an inserted epitope tag. The same expression pattern was also found in mice transgenic for the 16 kb construct GCTag, containing the complete coding region of the gene, including intragenic sequences, as well as 3 kb of upstream and 2 kb of downstream sequences. The CRABP-Tag transgene is expressed from these constructs in the outer layer of the midbrain, the hindbrain and the mantle layer of the spinal cord, in neural crest, limb buds, in the mesonephric mesenchyme, and in the frontonasal mass, i.e. all known CRABP-I expression sites in the embryo.

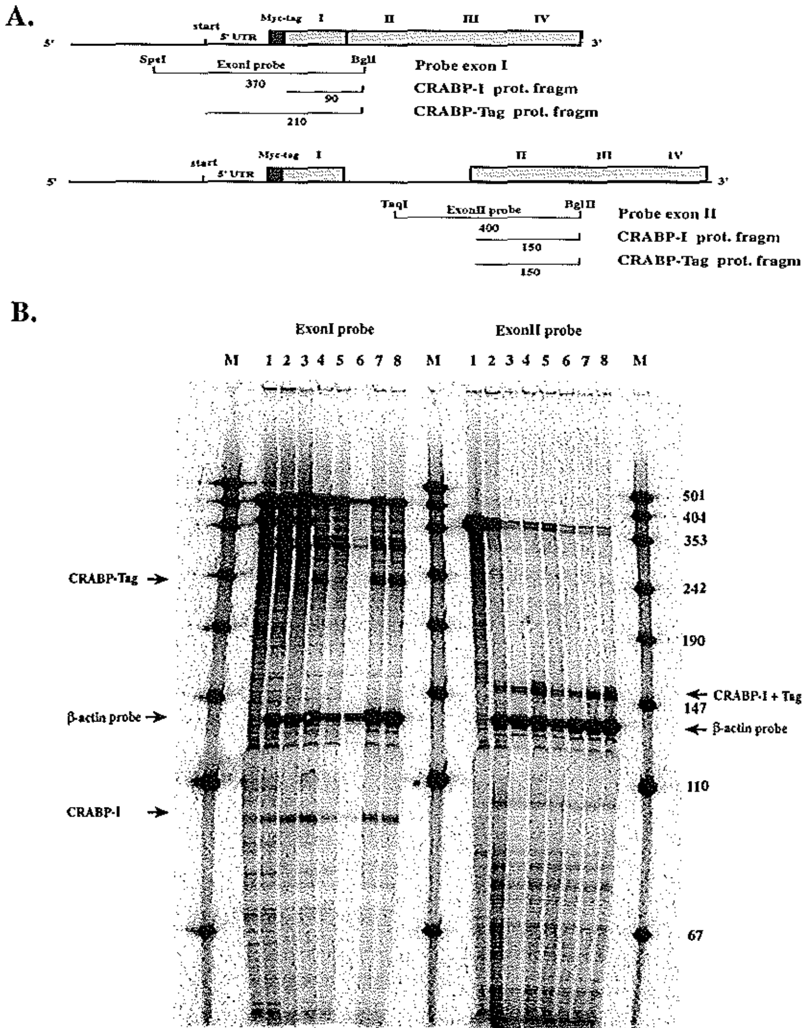


Figure 5: S1 analysis of the levels of CRABP-I and CRABP-Tag in transgenic embryos. (A) Schematic representation of the probes used for the S1 analysis. The exon I probe was derived from the construct pSalcDNATag (top line), in which the CRABP upstream region is linked to the cDNA in exon I. CRABP-I mRNA gives rise to a protected fragment of 90 basepairs after hybridisation and S1 digestion, while CRABP-Tag mRNA, due to the presence of the Myc-tag sequences in the probe, gives a 210 basepair protected fragment. Hybridisation with the exon II probe, derived from pECTag (bottom line), gives the same band of 150 basepairs with both CRABP-I and CRABP-Tag mRNA, and is indicative of the total level of CRABP present. The ß-actin probe is used as an internal control to correct for different levels of RNA between the lanes. (B) S1 gel, showing the overexpression of CRABP-Tag in the transgenic lines M4Tag4 and GCTag3. M=marker; 1, tRNA control; 2, wildtype 3x probe; 3, GCTag7; 4, M4Tag4; 5, M4Tag9; 6, wildtype; 7, GCTag3 heterozygote; 8, GCTag3 homozygote. The position of the CRABP-I, CRABP-Tag and ß-actin protected fragments is indicated.

In the limb bud we observe a proximo/distal gradient of the transgene with highest expression levels found distally, consistent with the expression observed by others (Dolle, 1989). However the existence of an anterior/posterior gradient is less clear. A gradient with the highest level anteriorly has been reported by some (Maden , 1988; Perez-Castro , 1989), while the absence of a gradient has been reported by others (Dolle , 1989; Ruberte , 1992). Our results support the observations of Dolle and Ruberte since we did not find consistent evidence for an antero/posterior gradient in the limb bud.

In some of the transgenic lines with M4Tag and GCTag expression of the transgene could not be detected in the frontonasal mass. These lines were shown by S1 analysis to have an overall low level of expression of the transgene compared to the endogenous gene (data not shown), and thus the expression in the frontonasal mass probably remained below detection level in those lines. In the lines that exhibit a high level of CRABP-Tag expression relative to the endogenous CRABP-I a clear expression of the transgene was found in the frontonasal mass. In all lines with M4Tag and GCTag the level of expression was lower than would be expected from the copy number of the transgene. This suggests the presence of an additional regulatory element that is involved in controling the level of expression.

Clearly the proximal promoter region of CRABP-I alone, as represented by the constructs XCTag or SCTag, is insufficient to drive expression of the gene. In summary, we conclude that all the cis-acting elements required for the regulation of the correct spatio-temporal expression of CRABP-I are located within a 16 kb fragment GCTag.

Distinct enhancers drive the expression of CRABP-I in neural and in mesenchymal/neural crest tissue

Further dissection of the construct GCTag revealed that the tissues that express CRABP-I during mouse embryonic development can be divided into two groups. Different sets of enhancers are used to drive the expression of CRABP-I in these tissues. Constructs containing the upstream region of GCTag, but lacking most downstream sequences, i.e. ECTag, show expression of the transgene in a neural subdomain of the CRABP-I expression sites. Constructs with the downstream region from GCTag, but lacking an upstream fragment, i.e. XHTag, drive expression of CRABP-Tag in a subdomain of CRABP-I which contains mesenchymal and neural crest cells. Expression of CRABP-I in the latter group, which contains neural crest cells, including those of the dorsal root ganglia and in the otic vesicle, and the mesenchyme in the mesonephric area and the limb bud, is apparently regulated via enhancer(s) located downstream of the second exon of the gene, as these cells express CRABP-Tag from the construct XHTag, but not from ECTag. Their identification in the future may provide a useful tool in the study of the development of the limb bud and neural crest.

The second group of cells that express CRABP-I during murine development is formed by the cells in the outer layer of the midbrain, the hindbrain and the ventral part of the mantle layer of the spinal cord. These cells show expression of the transgene in mice containing the construct ECTag. This neural CRABP-I enhancer must thus be localised in the 2 kb EcoRI/XhoI upstream fragment from -3200 to -1100 relative to the gene.

Overexpression of CRABP-Tag does not create an apparent phenotype.

Maintainance of physiological levels of RA is important for correct embryonic development. The CRABPs may serve a role in regulating the intracellular levels of RA in particular embryonic tissues, but their exact role is still unclear. The expression pattern of

CRABP-I in the embryo coincides with the structures that are most sensitive to RA excess (Vaessen, 1990). Whether this correlation of CRABP expression with the RA sensitive tissues is coincidental, or whether CRABP-I serves a role in the RA signalling mechanisms remains to be shown. Both CRABP-I and CRABP-I/CRABP-II deficient mice have been generated (De Bruin, 1994; Gorry, 1994; Lampron, 1995), but no apparent phenotype could be detected in these mice. Challenging these mice with RA stress conditions did not reveal any difference between normal and CRABP deficient mice (Lampron, 1995).

Since no apparent phenotype could be detected upon the functional deletion of the gene for CRABP-I, we have used the opposite approach to study the function of the gene in embryogenesis, i.e. the generation of transgenic mice that overexpress the gene within the confines of its endogenous expression domain. Both M4Tag and GCTag constructs contain the complete, tagged CRABP-I gene, and were shown to express the transgene in the correct expression domain, the transgenic lines for these constructs could potentially be overexpressing CRABP-tag within its endogenous expression domain. We first investigated whether the presence of this epitope tag interfered with the RA binding capacity of CRABP-I. We show here that in an *in vitro* RA binding assay the tagged CRABP protein is equally capable of binding retinoic acid as the wildtype CRABP-I, showing that this function of CRABP is not impaired by the presence of the Myc epitope. Next the level of expression of CRABP-Tag in the various transgenic lines was assessed by S1 analysis of the mRNA levels in transgenic embryos. We found that two of the transgenic lines have approximately a 3 to 5 fold higher levels of CRABP mRNA than wildtype embryos. We were however unable to detect any abnormalities in these mice. Embryos from the overexpressing lines do not show abnormal embryonic development. Reproduction appears unaffected and normal ratios of transgenic to non-transgenic offspring are obtained upon breeding. Thus, overexpression of CRABP-I does not lead to an apparent abnormal phenotype under normal laboratory circumstances. Based on the combined results from a number of studies the hypothesis has been put forward that the CRABPs serve a function in protecting the organism against environmental vitamin A stress situations. These CRABP-overexpressing transgenic mouse lines represent an interesting model system in which the putative function of CRABP-I under conditions of vitamin A excess or deficiency can be studied further.

In summary, we have shown that the expression pattern of CRABP-I during murine embryonic development consists of two separate expression domains, and that distinct enhancer elements are involved in the transcriptional regulation of the gene in these expression domains.

Acknowledgements

We are thankful to Lien Braam and her staff for animal husbandry, to Mirko Kuit for expert help with photography and to Jacky Guy and Elaine Dzierzak for critical reading of the manuscript. R. Poelmann and A. Gittenberger-de Groot are acknowledged for their support. D.A.K. and S.D. were supported by a grant from the Dutch Heart Foundation (NWO/NHS grant 900-516-096).

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CHAPTER V

MOLECULAR ANALYSIS OF THE CRABP-I NEURAL ENHANCER REGION

Molecular analysis of the CRABP-I neural enhancer region.

Part of this chapter was published in:

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Summary

The Cellular Retinoic Acid Binding Protein type I (CRABP-I) is believed to play a role in the retinoic acid (RA) signal transduction pathway. During mouse embryonic development CRABP-I is specifically expressed in the neural crest, branchial arches, limb bud, frontonasal mass and in certain structures in the central nervous system (CNS). All these structures are sensitive to aberrant retinoic acid (RA) concentrations. In the previous chapter we have shown that a 16 kb fragment surrounding the gene harbours all the elements that are needed for its correct spatiotemporal expression pattern. Upon further dissection of this fragment we have found that expression in the CNS is driven by elements in the upstream region of the gene, while expression in mesenchymal and neural crest tissue is regulated via element(s) located downstream of exon II of the gene. In this study we have further investigated the upstream region that is responsible for CRABP-I expression in the CNS. The presence of two distinct fragments in that region appears to be required for expression in the CNS, with neither of these fragments alone being able to drive correct expression of a reporter gene in transgenic mice. DNAseI footprinting analysis of the two upstream fragments revealed the presence of three protected sequence elements in one of the fragments, and one protected element in the other. One of these regulatory elements has the hallmarks of a novel RA response element, suggesting that CRABP-I expression in neural tissue can be directly modulated by RA via the RARs/RXRs. When oligonucleotides encompassing the protected elements were tested in an electrophoretic mobility shift assay using a nuclear extract from embryonic neural tissue, a number of retarded complexes were found on each of the four elements, indicating that a complex interplay of transcription factors is involved in the transcriptional regulation of CRABP-I during murine embryonic development.

Introduction

The Cellular Retinoic Acid Binding Protein type I (CRABP-I) is a small intracellularbinding protein, which forms part of the retinoic acid signal transduction pathway. The protein is thought to be involved in regulating the availability of retinoic acid (RA) for the nuclear RA receptors. CRABP-I shows a spatiotemporally specific expression pattern during embryonic development, with expression found in the tissues that are most sensitive to RA excess

(Vaessen, 1990). Expression is found in the central nervous system (CNS), the neural crest, the dorsal root ganglia, the limb bud and the frontonasal mass (Ruberte, 1991; Ruberte, 1992; Horton, 1995; Lyn, 1994). In the CNS the protein is expressed in the outer layer of the midbrain roof, in the hindbrain and in the mantle layer of the spinal cord.

We have studied the transcriptional regulation of the CRABP-I gene during embryonic development in transgenic mice. We showed in the previous chapter that a 16 kb construct, GCTag, is able to regenerate the complete expression pattern of endogenous CRABP-I. Deletional analysis of this construct revealed that the expression pattern of the transgenic CRABP can be split into two domains: a neural domain and a mesenchymal/neural crest domain. Expression in the mesenchymal/neural crest domain is driven by element(s) located downstream of exon II of the gene, while the upstream region of CRABP-I is responsible for the expression in the neural subdomain. In this chapter we have further studied this putative regulatory region by a deletional analysis in transgenic mice, followed by DNAseI footprinting and bandshift analysis of the identified enhancer fragments. Four sequence elements were thus identified. One of these elements is a novel RA response element, which would allow for modulation of CRABP-I expression by RA. The other three elements are all shown to bind multiple factors, suggesting that a complex interplay of transcription factors regulates the expression of CRABP-I during murine embryonic development.

Materials and methods

Constructs

A 5500bp genomic EcoR1 fragment containing exons I and II of the murine CRABP-I gene was subcloned into the vector pIC19H (Marsh, 1984), creating pDJ1. The LacZ reporter constructs were made by cloning the fragments EcoR1-Eco47III, Eco47III-MscI and EcoR1-XhoI from pDJ1 into the blunted SalI site in the vector p610Za, which contains the hsp68 minimal promoter, the LacZ gene and the SV40 polyadenylation signal (Kothary, 1989). This resulted in the constructs EE47hspZ, E47MhspZ (Z5) and EXhspZ (Z0) respectively. Microinjection constructs Z1 and Z2 were made from EE47hspZ. Construct DABhspZ (Z3) was made by deleting an AvrII/BglII fragment from EE47hspZ. Z4, Z6 and Z7 were made from EXhspZ (Z0). Construct DMXhspZ (Z8) was made by deleting an MscI/XbaI fragment from EXhspZ. The microinjection fragments Z8 and Z9 were derived from this construct. Deletion of a NheI/XbaI fragment from EXhspZ (Z0) was performed to create DNXhspZ (Z10). The fragments Z10 and Z11 were made from this construct.

Transgenic mice production and processing of the embryos

Microinjection was performed according to standard procedures (Hogan, 1994). For all other constructs primary embryos were collected at day 10.5 or 11.5 p.c.. For staining with X-gal embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM ethylene glycol-bis(beta-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.02% Nonidet P-40 (NP-40). Transgenic embryos were identified by Southern blot analysis of placenta DNA.

Embryos transgenic for LacZ constructs were stained for several hours or overnight at 37°C in the dark in a solution containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, 0.01% sodiumdeoxycholate, 0.02% NP40 and 0.1% 5-bromo-4-chloro-3-indolyl-ß-D-galacto-pyranoside (Xgal).

DNAse I footprinting analysis

The midbrain, hindbrain and spinal cord were excised from approximately 120 embryos of 11.5 days p.c.. Crude nuclear extracts were prepared from the excised tissue as described (Andrews, 1991), aliquoted and stored at -80°C. The fragments BglII/NheI, XbaI/XhoI and SacI/HincII corresponding to the -2280 to -2015, -1360 to -999 and -1171 to -986 upstream CRABP-I regions respectively, were end-labeled at either the 5' or the 3' end, purified on a polyacrylamide gel, and incubated with 4 to 40 mg of nuclear extract and 1 mg of poly(dI-dC) for 20 minutes at room temperature in a binding reaction containing 20 mM Hepes (pH 7.9), 8% glycerol (vol/vol), 40 mM KCl, 0.2 mM PMSF, 1.5 mM DTT, 0.08 mM EDTA, and 1.25 mM $MgCl_2$. After cooling on ice for one minute 0.5 to 2.0 mg of DNAse I (Boehringer) in 1 ml of 10mM Tris (pH 7.5) was added and the reaction was incubated on ice for 180 seconds. The assay was stopped by the addition of an equal amount of 1.2 M NaCl, 1% sodium dodecylsulphate (SDS), 30 mM EDTA and 30 mg yeast tRNA, and incubated with 20 mg of proteinase K for 1 hour. After phenol/chloroform extraction and ethanol precipitation the pellets were resuspended in 10 ml loading buffer containing 95% formamide, denatured for 5 minutes at 95°C and run on a 6% denaturing polyacrylamide gel, alongside G+A tracks of the same DNA (Maxam, 1980). Gels were dried, exposed to a phosphorescent screen for several hours and scanned on a PhosphorImager (Molecular Dynamics) using ImageQuant software.

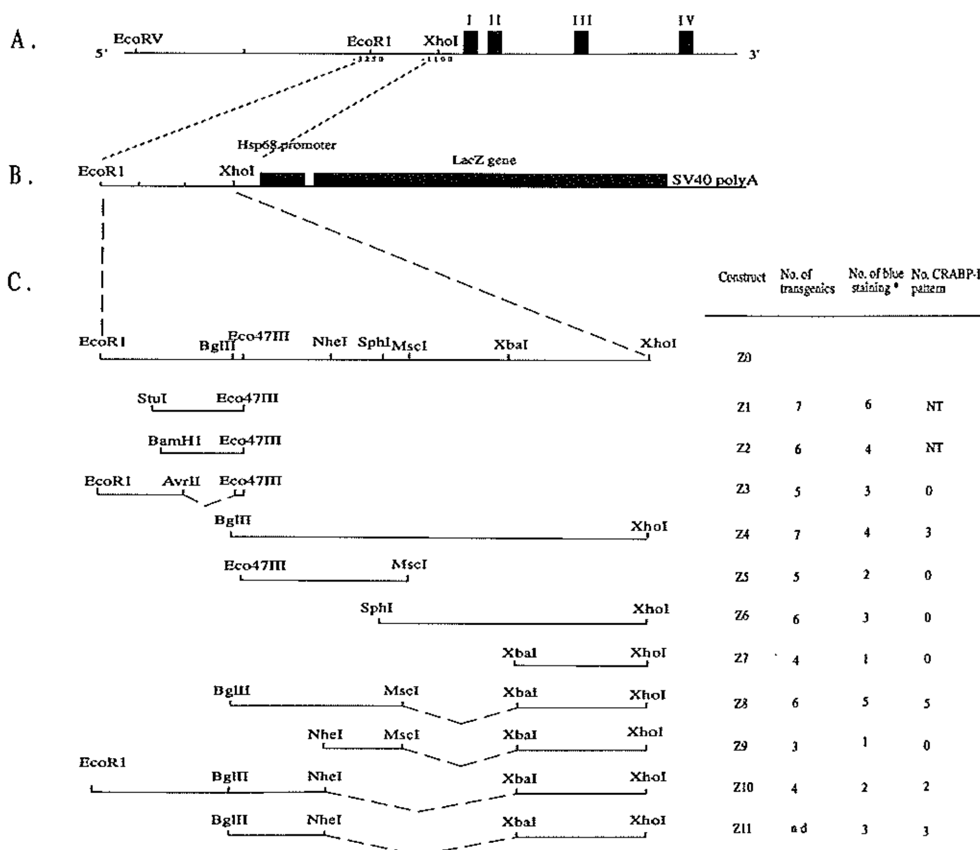
Electrophoresis Mobility Shift Assay

Oligonucleotides (Eurogentec), selected to encompass the protected areas from the DNase I footprinting assays, were 5' end-labeled with T4 polynucleotide kinase and (g-^{32}P)ATP (Sambrook, 1989) and annealed to their complementary partner oligos.

The following oligonucleotides were used as probes or competitors (coding strand sequences are listed; mutated nucleotides are indicated by lowercase in the oligos used as competitor): oligo XX1, GAATTTTACAACACCTGTGTCATGAGGAGTG; oligo Box-XX1.1, GAATTggcaccaACCTGTGTCATGAGGAGT; oligo Box-XX1.2 GAATTTTACAA-CAaagtttgCATGAGGAGT; oligo Box-XX1.3, GAATTTTACAACACCTGTGTacgtcttAGT; oligo RARE, AGGAAAAGTGACCTTTGGGGACCTCGAGCA; oligo RARE7, AGGAAA-AGTGACCTTTGGttcGGACCTCGAGCA; oligo RAREMut, AGGAAAAGgtcaagTTGGGG-ACCTCGAGCA; oligo RARED2, AGGAAAAGctgaagTTGGtacagaCGAGCA; oligo XX2, AGAAGGAATCCTGTCAATTCCGAGGAAAGTAATCTGCTTAGGACCT; oligo ß-RARE, CCGGGTAGGGTTCACCGAAAGTTCACTCG; oligo 2x AP-1, GAAACCTGCTGACTCA-GATGTCCTGAAACCTGCTGACTCAGATGTCCT; oligo 2x Sp1, AAATAGTCCCGCCCC-TAACTCCGCCCAT. Labeled double-stranded DNA (0.05 ng) was incubated at 20°C for 20 minutes with 4-6 mg of neural nuclear extract in the presence of 2 mg of poly(dI-dC) in a final volume of 10 ml, containing 5mM Tris.HCl pH 8.0, 1% Ficoll, 25 mM NaCl, 0.5 mM DTT and 0.5 mM EDTA. For each competition experiment a 100 fold excess of nonradioactive double stranded oligonucleotide was added. For the supershifts with oligo RARE antibodies against RARα (Ab9a), RARγ (Ab1γ1) and RXR were kindly provided by C. Rochette-Egly and P. Chambon (Rochette-Egly, 1991). After 15 minutes of incubation of the oligo with nuclear extract, 1 ml of ascite fluid antibody was added to the mixture and incubated for another 15 minutes. The DNA-protein complexes were analysed by electrophoresis on a 4% nondenaturing polyacrylamide gel (37.5/1 acrylamide/bis) in 0.5x TBE (25 mM Tris; 25 mM Boric acid; 1 mM EDTA) at room temperature. After electrophoresis the gel was dried and scanned on a PhosphorImager (Molecular Dynamics) using ImageQuant software.

Results

To define the cis acting sequences that direct the expression of CRABP-I in the central nervous system of the mouse embryo we have performed a deletional analysis of the upstream region of the gene. This region was defined as containing the putative CNS enhancer by the expression pattern found with the construct ECTag (see previous chapter). A number of reporter constructs containing the CRABP-I upstream region driving a LacZ reporter gene were made and analysed in transgenic founder embryos. These constructs are illustrated in Fig. 1. This led to the identification of two fragments, both of which appeared to be required to drive LacZ expression in the correct expression sites. Both fragments were subjected to DNAseI footprinting analysis. Four distinct cis-acting elements were defined, and subsequent bandshift analysis of these elements revealed the presence of multiple complexes on each of the elements.



Construct	No. of transgenics	No. of blue staining *	No. CRABP-I pattern
Z0			
Z1	7	6	NT
Z2	6	4	NT
Z3	5	3	0
Z4	7	4	3
Z5	5	2	0
Z6	6	3	0
Z7	4	1	0
Z8	6	5	5
Z9	3	1	0
Z10	4	2	2
Z11	nd	3	3

Fig. 1: Deletional analysis of the 5' region of CRABP-I. (A) Genomic map of the CRABP-I locus. (B) Map of construct EXp610Za (Z0), which contains 2.14 kb of CRABP-I upstream sequences driving the hsp68 minimal promoter, the LacZ gene and the SV40 polyadenylation signal. (C) microinjection fragments derived from construct EXp610Za. * No. of blue staining embryos indicates both embryos with CRABP-I CNS staining pattern and embryos with staining due to postion effect (see text). NT = particular band of X-gal staining cells in the bottom third of the spinal cord of transgenic embryos. nd = not determined.

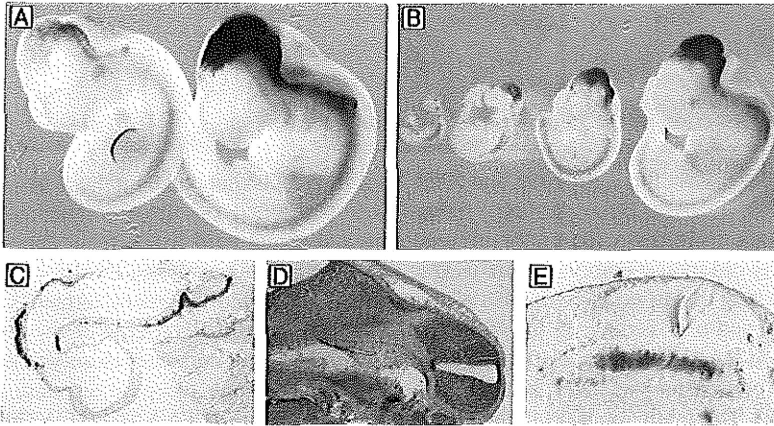


Fig. 2: X-gal staining of LacZ transgenic embryos. (A, B) Transgenic embryos for construct Z4 show LacZ expression in the midbrain, the hindbrain, the cranial nerves and spinal cord. (C) Cross section of an X-gal stained embryo to indicate the staining pattern in the outer layer of the mid- and hindbrain. (D) Section of a transgenic embryo showing LacZ staining in the mantle layer of the spinal cord. (E) Cross section of the spinal cord of a transgenic embryo with construct Z2, showing a band of X-gal staining at the ventral side, peculiar to only constructs Z1 and Z2.

Deletional analysis of the neural enhancer fragment

To demonstrate that the upstream element directing the neural expression found with ECTag can also act as an independent enhancer element we cloned the 2 kb EcoR1/XhoI upstream fragment in the vector p610Za, resulting in construct EXhspZ (Z0, Fig. 1). In the vector p610Za the LacZ gene is driven by a mouse heat-shock promoter (hsp68). It has been demonstrated that this hsp68-LacZ construct does not give any constitutive expression in transgenic mouse embryos, making it a useful vector for testing the presence of regulatory elements in heterologous sequences (Kothary, 1989). The construct Z4 was found to direct the expression of the LacZ gene in the neural CRABP-I expressing cells in the midbrain, hindbrain and spinal cord (Fig. 2), consistent with the expression pattern found with the ECTag construct. A series of constructs was made by deleting various fragments from construct Z0 (Z1 to Z11, Fig. 1). The results of these experiments are compiled in figure 1. The fragments BglII-NheI and XbaI-XhoI are found to be important for the expression of LacZ in the neural tissue of transgenic mice. The presence of both these fragments is required since constructs Z8, Z10 and Z11 show correct expression of the LacZ gene, whereas the constructs Z5, Z6, Z7 and Z9 do not. For each of these constructs one or more embryos were obtained showing LacZ expression due to a position effect. In these embryos X-gal staining was found in ectopic tissues, which varied between independent primary embryos injected with the same construct. The constructs Z1 and Z2 also appear to contain a regulatory element since embryos transgenic for these constructs show

consistent expression of the LacZ reporter gene in a specific band in the spinal cord (Fig. 2E). The significance of this fragment for regulation of the endogenous CRABP-I gene is unclear, as the site of expression of the LacZ reporter gene is not a site of CRABP-I expression at this developmental stage. It may be an artefact created by placing the fragment in an unnatural environment.

Molecular dissection of the neural CRABP-I enhancer

To further dissect the BglII-NheI and XbaI-XhoI fragments that make up the neural enhancer of CRABP-I, DNAse I footprinting analysis was performed on these fragments. Nuclear extracts, made from dissected midbrain, hindbrain and spinal cord tissue from approximately 120 11.5 day old mouse embryos, were used in these footprinting analyses. DNase I footprinting with this extract identified three distinct elements in the XbaI-XhoI fragment, termed XX1, XX2 and RARE, and one element in the BglII/NheI fragment, termed BN2. One of the footprinted areas, termed RARE, contains a direct repeat (DR) with half-site sequences closely matching the AGGTCA consensus sequence found in other nuclear hormone receptor response elements (Leid, 1992). The motif found in the RARE element is AGGTCCTTAAAGGTCA (in reversed orientation), and has a spacing of 4 basepairs between the half-sites. A DR-4 is normally associated with binding of a thyroid hormone (TR)/retinoid X receptor (RXR) heterodimer (Umesomo, 1991), but can also be a response element for RAR/RXR heterodimers depending on the context of the element (Mader, 1993; Mader, 1993; Nagpal, 1993). Alternatively the element could be a binding site for the orphan receptor NGFI-B, having a perfect match to the reported AAAGGTCA consensus binding site for this factor. To confirm that the protected areas that were identified in the footprinting assays indeed correspond to transcription factor binding sites we performed bandshift experiments with oligonucleotides covering these protected areas. All four elements (oligos BN2, XX1, XX2 and RARE) showed a number of retarded bands on the gel, that were specific since competition with a 100 fold excess of nonradioactive oligo (self) abolished these retarded complexes, whereas competition with a 100 fold excess of a non-related oligo did not affect the intensity of these retarded bands. The band indicated by arrow 2 in fig. 4, which is retarded in the bandshift assay with oligo XX1, is considered aspecific as it also appears when unlabeled oligo XX1 (self) is added as competitor.

Retarded complexes formed with oligo RARE were very abundant compared to those formed with the other oligonucleotides. Competition experiments with radioactively labeled oligo RARE and a 100-fold excess of unlabeled oligonucleotides that were mutated in one or both of the half-sites, or in which the spacing was changed from 4 nucleotides to 7 nucleotides, were carried out. These showed that mutating just one of the half-sites (oligo RAREMut) has no or very little effect on binding of the factor(s) to this oligo because this mutated oligo could still effectively compete out the labeled oligo RARE. The half-site mutated in the oligo RAREMut is the site resembling a NGFI-B recognition site, and since complete competition is found with this oligo it seems unlikely that NGFI-B is the factor binding this element. Changing the spacing between the half-sites (oligo RARE7) from 4 to 7 resulted only in a slight loss of competition ability, indicating that the spacing plays a minor role in the binding properties of this element. However when both half-sites were mutated (oligo RARED2) the ability to compete was lost completely. Competition with the DR-5 RARE from the RARβ gene was also effective, indicating that the factor binding to the CRABP-I response element could be a RAR/RXR heterodimer. We tested this hypothesis by adding antibodies against RARα, RARγ and RXR (all

isoforms) in the bandshift, and found that these antibodies could indeed supershift the complex formed on the putative RARE element.

Competition experiments were also performed on the other elements. Since the sequence of the element XX1 does not resemble the consensus recognition sequence of any known transcription factor we designed three systematically mutated oligonucleotides (oligos BoxXX1.1, BoxXX1.2 and BoxXX1.3). The mutation in BoxXX1.3 did not interfere with its ability to compete with labeled oligo XX1. Oligo BoxXX1.2 had completely lost the ability to compete, indicating that the binding sites are located in the mutated part of the oligo, i.e. in the CCTGTGT sequence, or at least comprise part of this sequence. The oligo BoxXX1.1 appears to compete for some of the retarded bands, but has lost the capability to compete for one of those bands (indicated by arrow number 4 in fig. 4). Since this band is also lost in the competition experiment with oligo BoxXX1.2 the recognition sequence for this particular factor is expected to overlap the sequences mutated in these oligos. The band indicated by arrow number 5 could be AP-1 as the intensity of this band is much reduced when an AP-1 oligo was added as competitor. The identity of other factors binding to the XX1 element remains at this stage unknown. The binding of these factors to XX1 is however tissue specific as is shown by the fact that the retarded complexes 1 and 4 are not found with extracts from MES-1 or MEL cells.

The element XX2 shows a shift which is largely competed out by addition of an oligo containing two consensus AP-1 sites, suggesting that this element also binds an AP-1 like factor. The retarded complexes found on the element BN2 are reminiscent of the complexes found on a regulatory element found in the Thy1 promoter (Spanopoulou, 1991). However, no competition is found with oligos containing Sp1 or AP-1 binding sites.

Interestingly, during the footprinting analysis of the BglII/NheI fragment we discovered a difference between the published sequence of this CRABP-I upstream region and our own sequence, located exactly in the BN2 element. The importance of the differing residues is shown by the fact that the oligo BN1, containing the published sequence plus an additional mutation, does not give a bandshift, and is also unable to compete BN2.

Discussion

The CRABP-I gene shows a very specific expression pattern during development. We have investigated the molecular mechanisms that are involved in the regulation of this expression pattern during murine embryonic development. We have previously shown that multiple sets of enhancer elements are employed by the gene (see Chapter IV). The complete spatial and temporal expression pattern of the gene is reproduced in transgenic mice with a 16 kb construct GCTag. Further dissection of that construct revealed that the tissues that express CRABP-I during mouse embryonic development can be divided into two groups. Different sets of enhancers are used to drive the expression of CRABP-I in these tissues. Constructs containing the downstream region of the gene drive expression of a CRABP-Tag reporter gene in a subdomain of CRABP-I which contains mesenchymal and neural crest cells. Constructs containing only the upstream region of CRABP-I show expression of the transgene in a neural subdomain of the CRABP-I expression domain. Expression of CRABP-I in this neural domain is found in the cells in the outer layer of the midbrain, the hindbrain and the ventral part of the mantle layer of the spinal cord. As no expression was found with constructs containing only the

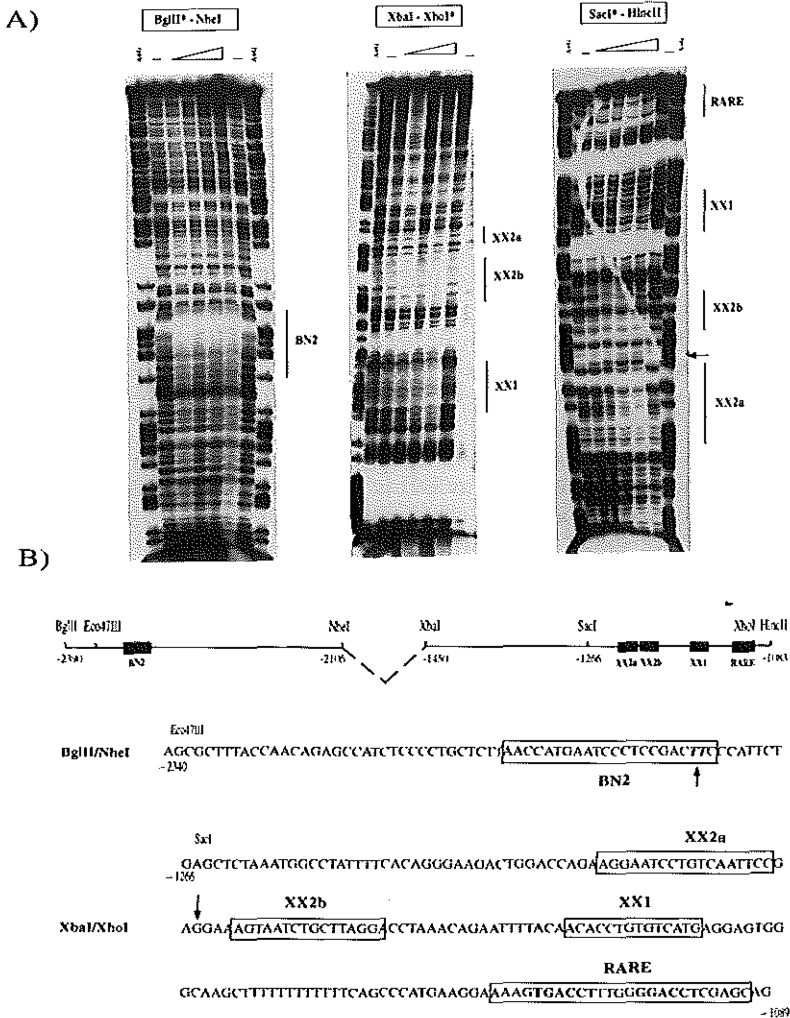


Fig. 3: (A) DNAse I footprinting analysis of the BglII/NheI and XbaI/XhoI fragments, which are the two fragments required for CRABP-I expression in neural tissue. The BglII/NheI fragment spans the region from -2400 to -2100 and the XbaI/XhoI fragment spans the region from -1390 to -1000 relative to the start of the coding region of the gene. The fragment used in each of the assays is indicated above the gel with the labeled end marked by an asterisk. The labeled fragments were incubated with 5, 10 , 20 or 40 mg of nuclear extract prepared from excised spinal cords, mid- and hindbrains from 11.5 days p.c. embryos, before digestion with DNAseI. Unprotected DNA is shown by -. A G+A Maxam and Gilbert sequence reaction is run along with each of the assays. The regions protected by the nuclear extract are indicated as **BN2, XX1, XX2a, XX2b** and **RARE**. The arrow indicates a hypersensitive site. (B) Map of the protected elements on the two fragments. Nucleotide sequences of the footprint containing parts of the BglII/NheI and XbaI/XhoI fragments. The regions that show a footprint in (A) are indicated by boxes. The two T residues in italics in element **BN2** indicate that these residues differ from the published sequence. The arrow between **XX2a** and **XX2b** indicates the position of the hypersensitive site. The region **RARE** contains a DR4 RA response element, of which the half-sites are indicated in bold face.

first 1kb upstream of the gene, we concluded that a neural enhancer for the CRABP-I gene must thus be localised in the 2 kb EcoRI/XhoI upstream fragment from -3200 to -1100 relative to the gene.

We show in this study that this region can act as an independent neural enhancer on heterologous promoters, since it is also able to drive LacZ expression in the same neural tissue when cloned into a hsp68LacZ vector (Z4, Fig.1). We next set out to closer define the cis-acting elements that are responsible for the neural component of the CRABP-I expression pattern by making a series of deletion mutants (Z5-Z11, Fig.1). The analysis of the constructs Z5 to Z11 shows that the neural enhancer maps to two fragments of 270 and 350 basepairs respectively. The presence of both of these fragments is required since none of the constructs Z5, Z6, Z7 and Z9 expressed the LacZ reporter gene in the correct neural tissue. Embryos with variable ectopic expression due to position effect were obtained with each of these constructs. This shows that at least some of the injected fragments of each construct landed in a transcriptional competent chromatin area. The constructs Z1 and Z2 which contain more upstream sequences from the CRABP-I promoter region were also able to drive expression of the LacZ reporter gene in transgenic embryos. However, the LacZ expression with these constructs was consistently found in a particular band throughout the thickness of the spinal cord, at approximately one third from the ventral side (Fig. 2). We are at present uncertain about the significance of this element for normal CRABP-I expression, since no endogenous CRABP-I expression is detected at that particular site in the spinal cord at this developmental stage. It is however reminiscent of CRABP-I expression found at later developmental stages in the commissural neurons of the spinal cord (Maden, 1992; Ruberte, 1992). However, it may also be an artefact caused by taking the element out of its normal environment, resulting in the ectopic activation of the element.

Specific cis-acting elements are required for CRABP-I neural enhancer activity in the developing nervous system

DNAse I footprinting analysis of the two upstream fragments revealed 4 protected regions, that were termed BN2, XX1, XX2 and RARE. Bandshift assays with oligonucleotides encompassing the sequences that were protected in the footprinting assays show that indeed protein/DNA complexes are formed on these elements. The element RARE contains a nearly perfect consensus recognition site for nuclear hormone receptors (Leid, 1992) consisting of a direct repeat (DR) with a spacing of 4 nucleotides (DR4). A DR4 element is usually indicative for binding of thyroid hormone receptor/retinoid X receptor heterodimers (TR/RXR) (Umesomo, 1991), but depending on the context of the repeat binding of RAR/RXR heterodimers to certain DR4 elements has also been found (Nagpal, 1992; Mader, 1993). A DR4 element is part of a complex response element in the lamininB1 gene, which is induced by RA in F9 teratocarcinoma cells (Vasios, 1989). We have used antibodies against RARa, RARg1 and RXR(all isoforms) to show that the complex binding to the putative CRABP-I RARE contains both RARs and RXRs, and thus is most likely an RAR/RXR heterodimer. This is the first identification of an RA response element in the promoter of the CRABP-I gene. It is however not surprising considering that the genes of almost all other proteins involved in the RA signal transduction pathway contain RAREs in their promoters. Upregulation and anteriorisation of CRABP-I expression has been found in the developing nervous system of mouse embryos after

Fig. 4: (A) Bandshift assays with oligonucleotides encompassing the sequences that were found to be protected in the DNAseI footprinting assay. The radioactively labeled oligonucleotide used as probe in each experiment is indicated above the gel. A 100-fold excess of the following unlabeled competitors was used in the lanes indicated: a. oligo BoxXX1.1, b. oligo BoxXX1.2, c. oligo BoxXX1.3, d. oligo 2x Sp1, e. oligo 2x AP-1, f. oligo BN1. The arrows on the left of the panel with oligo XX1 indicate specific complexes. The band indicated by arrow 2 is considered aspecific as it also appears when unlabeled oligo XX1 (self) is added as competitor. (B) Bandshift assay on the putative CRABP-I retinoic acid response element (RARE). An labeled ds oligonucleotide from the wild-type CRABP-I RARE element was incubated with 3 mg of embryonic neural nuclear extract, in the presence or absence of various mutated oligonucleotides (a,b,c) or the RARE element from the RARß2 promoter (d). a. oligo RARE7, b. oligo RAREMut, c. oligo RARED2, d. oligo ß-RARE. Addition of antibodies against RARa, RARg and RXR (all isoforms) resulted in a supershift of the retarded complexes. The panel with the labeled RARß2 RARE oligonucleotide as probe (ßRARE) is included as a control, as this oligo is known to bind RAR/RXR heterodimers and the antibodies have been shown to be effective on these complexes.

RA treatment, when the RA was administered within a particular developmental time period (7-8.5 days p.c.) (Leonard , 1995). An upregulation of CRABP-I expression by RA in P19 cells has also been reported, but was considered to be an indirect effect as protein synthesis was found to

be required (Wei , 1989). Recently it has been shown that CRABP-I is upregulated in AB1 cells after treatment with RA at low concentrations, which are in the concentration range that is found in the embryo, but that this effect is abolished at higher RA concentrations (Chen, 1996). This may explain why in some studies no upregulation of CRABP-I was detected after treatment of embryos with an excess RA (Harnish, 1992).

One of the factors binding to the elements XX1 and XX2 appears to be AP-1, as an oligo containing two AP-1 sites competed some of the complexes formed on these elements. Both elements contain sequences that resemble an AP-1 recognition site. The other factors binding to XX1, XX2 and BN2 appear more difficult to identify. The bandshift with the element BN2 resembles the pattern of retarded complexes found with a regulatory element found in the promoter of the Thy1 gene (Spanopoulou, 1991). Some of the retarded bands found with that element were shown to be caused by binding of Sp1. However, competition with an Sp1 binding sites containing oligo did not inhibit the formation of any of the retarded bands with BN2. Thus the factors binding to BN2 and XX1 remain unidentified. The elements do not contain recognisable consensus sequences for binding sites of known transcription factors. Thus the sequence of the binding sites may have diverged from the consensus or the elements may contain binding sites for novel transcription factors. Two unidentified complexes on the XX1 element are tissue specific, since neither of them is formed with nuclear extract from CRABP-I expressing MES-1 cells or non-expressing MEL cells.

The elements that have been identified in this study are located on different fragments from the ones that were recently reported to be important for expression of CRABP-I in 3T6 and P19 cell lines (Wei, 1996). In our own studies we have also found that the expression of CRABP-I in the highly expressing cell lines MES1 and Tera2 is regulated differently to that in mouse embryos, since the fragments that direct CRABP-I expression in the mouse embryo are unable to drive expression of the chloramphenicol acetyltransferase (CAT) gene in those cells in transient transfection assays. This discrepancy shows that, at least in the study of the CRABP-I promoter region, cell lines are inadequate as a model for complex tissues *in vivo*. It is possible that the cell lines that were used are not a good representation of the tissues that express CRABP-I in the mouse embryo. Bandshift experiments with oligonucleotide XX1 indeed have shown that specific retarded complexes that are formed with the nuclear extract from excised neural tissue, are absent when MES-1 nuclear extract is used, indicating that the element XX1 binds different regulatory factors in different tissues or cell lines. In addition it is very well conceivable that the local chromatin environment forms an important factor in the spatiotemporal specific regulation of the CRABP-I gene, and this factor is clearly not taken into account in transient tranfection assays.

In summary, we show in this paper that the expression pattern of CRABP-I during murine embryonic development consists of two separate expression domains, and that distinct enhancer elements are involved in the transcriptional regulation of the gene in these expression domains. The expression of CRABP-I in the neural subdomain involves a complex interplay of regulatory factors at multiple enhancer elements. One of these elements is a RA response element with a 4 basepair spacing (DR-4) which is shown to bind RARs and RXRs, presumably as heterodimers, allowing for modulation of CRABP-I expression by its own ligand.

Acknowledgements

The antibodies against RARα, RARγ and RXR were kindly provided by C. Rochette-Egly and P. Chambon. The oligo ßRARE was kindly provided by G. Folkers. Gaetano Zafarana is gratefully acknowledged for helpful discussion and for providing the MEL cell extract. We are thankful to Lien Braam and her staff for animal husbandry, to Mirko Kuit for expert help with photography and to Jacky Guy for critical reading of the manuscript. D.A.K. and S.D. were supported by a grant from the Dutch Heart Foundation (NWO/NHS grant 900-516-096).

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CHAPTER VI

Cloning and sequencing of the CRABP-I locus from chicken and pufferfish; Analysis of the promoter regions in transgenic mice.

Cloning and sequencing of the CRABP-I locus from chicken and pufferfish; Analysis of the promoter regions in transgenic mice.

Dirk A. Kleinjan[©], Sylvia Dekker, Jacqueline A. Guy and Frank G. Grosveld*. Cloning and sequencing of the CRABP-I locus from chicken and pufferfish; Analysis of the promoter regions in transgenic mice. *Transgenic Research*, in press.

Abstract

Retinoic acid (RA), a derivative of vitamin A, is an important molecule for development and homeostasis of vertebrate organisms. The intracellular retinoic acid binding protein CRABP-I has a high affinity for RA, and is thought to be involved in the mechanism of RA signalling. CRABP-I is well conserved in evolution and shows a specific expression pattern during development, but mice made deficient for the protein by gene targetting appear normal. However, the high degree of homology with CRABP-I from other species indicates that the protein has been subject to strong selective conservation, indicative of an important biological function. In this paper we have compared the conservation in the expression pattern of the mouse, chicken and pufferfish CRABP-I genes to further substantiate this argument. First we cloned and sequenced genes and promoter regions of the CRABP-I genes from chicken and the Japanese pufferfish, *Fugu rubripes*. Sequence comparison with the mouse gene did not show any large blocks of homology in the promoter regions. Nevertheless the promoter of the chicken gene directed expression to a subset of the tissues that show expression with the promoter from the mouse gene. The pattern observed with the pufferfish promoter is even more restricted, essentially to rhombomere 4 only, indicating that this region may be functionally the most important for CRABP-I expression in the developing embryo.

Keywords: CRABP-I, chicken, pufferfish, promoter region, transgenic mice.

Introduction

Retinoic acid (RA) exerts a wide variety of effects on vertebrate development, cellular differentiation and homeostasis. Both excess and deficiency of RA during embryonic development result in a spectrum of congenital malfomations (Wilson, 1953; Kochhar, 1967; Lammer, 1985). The effects of RA are mediated at the molecular level via two classes of proteins, the retinoic acid receptor family and the retinoic acid binding proteins. The retinoic acid receptors are nuclear ligand-inducible transcriptional regulators belonging to the nuclear hormone receptor superfamily (Leid, 1992; Mangelsdorf, 1995; Chambon, 1996). The cellular retinoic acid binding proteins (CRABP-I, CRABP-II) are small binding proteins with high affinity for all-trans RA. In the adult organism CRABP-I is widely expressed, whereas CRABP-II expression is restricted to the skin. Both proteins show spatio-temporally specific expression patterns during embryonic development (Dolle, 1990; Ruberte, 1991; Maden, 1992; Ruberte, 1992; Lyn, 1994). Expression of CRABP-I is found in those tissues that are most sensitive to excess of RA, notably the developing central nervous system, the neural crest, limb bud mesenchyme and mesenchyme in the frontonasal mass and branchial arches (Vaessen, 1990).

Although the protein is mainly localised in the cytoplasm, recent reports have shown that in certain cell types CRABP-I can be found in the nucleus, suggesting a possible role in nuclear import of RA (Gustafson, 1996). A number of other roles have been suggested: It could act as a cytoplasmic buffer, protecting sensitive cells against excess of RA, it might be involved in the metabolism of RA to polar metabolites, or it could, under conditions of vitamin A shortage, function to sequester RA in the cells that are most dependent on it (Donovan, 1995; Napoli, 1996). However none of these roles have been satisfactorally proven. Moreover, mice deficient in CRABP-I or both CRABP-I and II are essentially normal, showing that CRABP-I is dispensable for normal development, at least under laboratory conditions (de Bruijn, 1994; Gorry, 1994; Lampron, 1995). In sharp contrast to this stands the fact that CRABP-I is highly conserved in evolution, which would indicate that it has functional importance for the organism. Bovine and murine CRABP-I are completely homologous at the amino acid level, and differ in only one out of 136 amino acids from human CRABP-I (Sundelin, 1985; Vaessen, 1989; Astrom, 1991). From a partial chicken cDNA it can be seen that chicken and mouse CRABP also share a high degree of homology at the amino acid level (Vaessen, 1990). Thus it appears that, even though the protein is apparently dispensable for mice under laboratory conditions, even a small change in the sequence of the protein is evolutionary unfavorable. Here we further substantiate this point with the cloning and sequencing of the CRABP-I genomic loci from chicken and the Japanese pufferfish, *Fugu rubripes*. Sequence analysis of the promoter regions from both these species was carried out, with the aim of identifying conserved promoter/enhancer elements. We have recently shown that the expression of CRABP-I in the mouse embryo is regulated via distinct sets of enhancer elements (Kleinjan, 1997). The expression pattern of CRABP-I in the chicken embryo is similar to that in the mouse, suggesting that the same cis-acting transcription elements may be involved in the regulation of expression. Although no information is available on the expression of CRABP-I in the pufferfish, its small genome size makes it a useful model species to look for conserved regulatory elements (Brenner, 1993; Aparicio, 1995). Here we report the cloning and sequencing of over 3 kb of upstream promoter sequences from both of these species. These sequences were tested for transcriptional regulatory activity in transgenic mice, where they are shown to drive specific patterns of expression of a LacZ reporter gene in the hindbrain region. The sites of LacZ expression form a subdomain of the murine CRABP-I expression pattern, with the extent of the subdomain decreasing with increasing evolutionary distance between the species.

Materials and methods

Cloning, mapping and sequencing of CRABP-I from *Fugu rubripes*.

The clones A176 and K1328 were isolated from a gridded ICRF *Fugu rubripes* genomic cosmid library, based on the cosmid vector Lawrist4 (C. Burgtorf and H. Lehrach, Berlin). The library was screened using a ^{32}P-labelled probe generated from the 170 basepair TaqI fragment from mouse CRABP-I (Vaessen, 1990), in a hybridisation mixture containing dextran sulphate (Sambrook, 1989). Duplicate nylon filters were hybridised at 56°C for 16 hours, washed twice for 20 min. in 3xSSC/0.1%SDS, and once in 1xSSC/0.1%SDS (1xSSC=150mM NaCl/15mM sodium citrate, pH 7) at 56°C, and autoradiographed. Positive clones were picked and grown for DNA isolation. The cosmid clones A176 and K1328 were used to map the *Fugu* CRABP-I locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot

transfer, and hybridisation were performed as described (Sambrook, 1989). Various fragments were subcloned into pBluescriptII for sequencing. The genomic sequences of the upstream region and surrounding the exons have been submitted to the EMBL database under accession numbers Y12240, Y12241 and Y12242.

Cloning, mapping and sequencing of CRABP-I from chicken, *Gallus gallus*.

The phage clones ChCRABP 8 and ChCRABP 17 were isolated from an adult Leghorn Chicken liver EMBL-3 SP6/T7 genomic library (Clontech) using a 310 bp Chicken CRABP-I partial cDNA clone as probe (Vaessen, 1990). Hybridisation of the library was performed as described for the *Fugu* library, except that in this screening the filters were hybridised and washed at 65°C. Positive plaques were rescreened and positive clones were used to isolate DNA. Mapping and sequencing of the Chicken CRABP-I locus was done as described for *Fugu* CRABP-I. Genomic sequences of the upstream region, and surrounding the three sequenced exons have been submitted to the EMBL database under accession numbers Y12243 and Y12244.

Production and analysis of transgenic embryos:

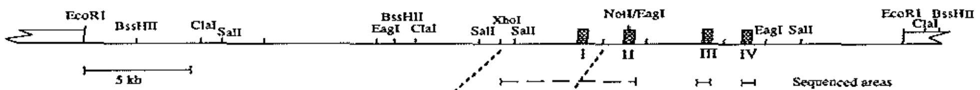
The microinjection constructs containing the mouse, chicken and pufferfish promoter regions were made as follows: A 5500bp genomic EcoR1 fragment containing exons I and II of the murine CRABP-I gene was subcloned and in the resulting plasmid the C residue at position +4 of the coding sequence was changed to a G by site-directed mutagenesis, thus creating an NcoI site at the translational start site of the gene. A 30 bp sequence coding for a 10 amino acid peptide tag derived from the human c-myc proto-oncogene (Evan, 1985) was cloned into this site, to create pDJTag. pECTag was constructed by linking the cDNA sequences for exons II,III and IV in frame to exon II of pDJTag. The mouse promoter/LacZ reporter construct was created by inserting an EcoRI/XhoI upstream fragment from the murine CRABP-I gene into the vector p610Za (Kothary, 1989), resulting in EXp610Za. The microinjection fragment BXhspZ was isolated by BglII/Asp718 digestion of EXp601Za.

The chicken promoter/reporter construct Ch1 was created by subcloning a 6 kb EcoRI fragment containing exons I and II into pBluescriptII KS. From this plasmid a 3.6 kb NotI fragment containing the promoter region, exon I, intron I and part of exon II was excised and ligated into the IRES-LacZ vector ßGeo (Mountford, 1994). The *Fugu* promoter reporter/construct Fu1 was made by subcloning of a 4.2 kb SpeI fragment, containing the promoter region as well as exon I and part of intron I, from cosmid A176 into pBluescriptII KS. From the resulting construct a 3.8 kb XhoI fragment was subcloned into the SalI digested p610Za vector.

Microinjection of mouse oocytes was performed according to standard procedures (Hogan, 1994). Primary embryos were isolated from pregnant females at day 10.5 or 11.5 p.c.. For LacZ staining the embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2mM $MgCl_2$, 5mM ethylene glycol-bis(beta-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.02% Nonidet P-40 (NP-40) in PBS (Phosphate Buffered Saline) for 60-90 minutes, washed with PBS containing 0.02% NP40, and stained for several hours or overnight at 37°C in the dark in a solution containing 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_2O$, 2mM $MgCl_2$, 0.01% sodiumdeoxycholate, 0.02% NP40 and 0.1% 5-bromo-4-chloro-3-indolyl-ß-D-galacto-pyranoside (Xgal).

Embryos transgenic for the ECTag construct, containing the Myc-tag as a reporter, were fixed for 1 hour in 35% methanol, 35% acetone, 5% acetic acid. After dehydration in an ethanol/xylene series the embryos were embedded and sectioned. The sections were rehydrated in ethanol/xylene. Aspecific binding was blocked by pre-incubating the sections in PBS/Tween20 (0.05%) containing 2% NGS (normal goat serum). After three washes with PBS/Tween20 the sections were incubated overnight at 4°C with a monoclonal antibody agains the Myc epitope in a 1:100 dilution in PBS containing 1% BSA and 0.05% Tween20. After washing the sections were incubated with an alkaline phosphatase conjugated goat anti-mouse antibody for several hours at room temperature. The sections were washed and exposed to NBT/BCIP as described elsewhere (Sambrook, 1989). Finally, the sections were dehydrated and mounted in glycerol/PBS.

A.



B.

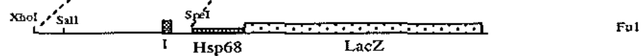


Fig. 1: (A) Map of cosmid A176, containing the CRABP-I locus from the Japanese puffer fish, *Fugu rubripes*. The 4 exons are indicated as filled boxes. The regions that have been sequenced are indicated by the broken line underneath the map. (B) Fu1 is the microinjection fragment that has been used to assess the puffer fish promoter region in transgenic mice. It contains 3 kb of upstream sequences, exon I and part of intron I driving a hsp68 promoter/LacZ reporter cassette.

Results

Cloning and characterisation of pufferfish CRABP-I

Using a 170 bp TaqI fragment encoding part of the mouse CRABP-I cDNA as a probe we have screened a gridded *Fugu rubripes* genomic ICRF cosmid library (C. Burgtorf and H. Lehrach, Berlin) under reduced stringency conditions. This resulted in the isolation of a small number of positive clones. Restriction mapping, hybridization with other probes and sequencing of a small fragment revealed that the isolated clones could be categorised into two groups derived from two different loci. Partial sequencing and hybridisation with additional mouse CRABP-I cDNA probes identified the clones A176 and K1328, representing one group, as *Fugu* CRABP-I. The other group of clones, possibly representing CRABP-II, was not further characterised. A restriction map of cosmid A176 is shown in Fig. 1A. As all other CRABPs, the gene contains four exons. The overall size of the gene is approximately 9 kb, and therefore no smaller than the mouse gene.

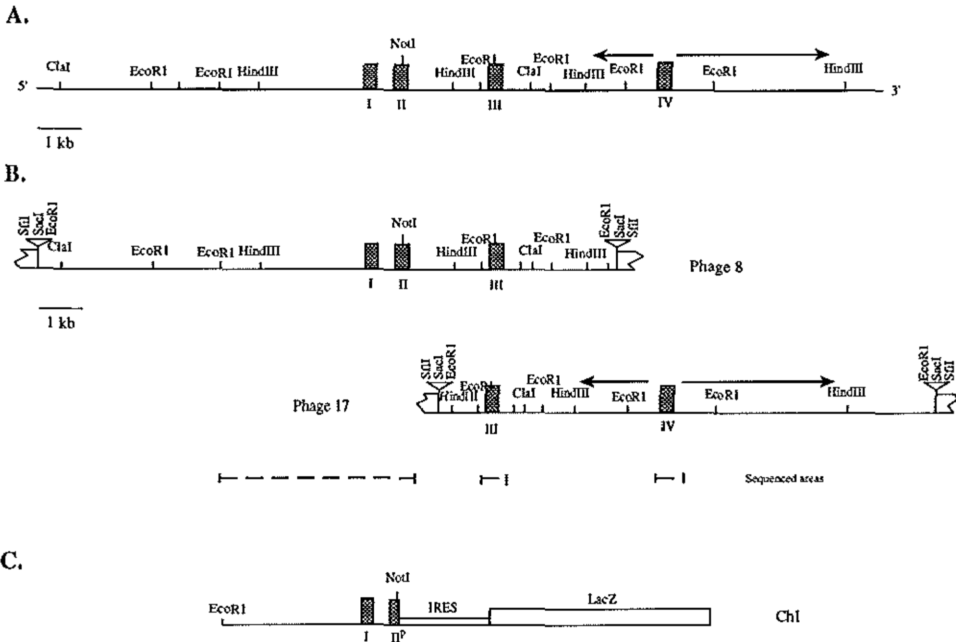


Fig. 2: (A) Genomic organisation of the Chicken CRABP-I locus. (B) Phages 8 and 17 were obtained from screening a chicken genomic library. Phage 8 contains exons I, II and III, as well as 8 kb upstream sequences. Phage 17 contains exons III and IV of CRABP-I plus downstream sequences. The broken line underneath the map of the phages indicates the regions that have been sequenced, which include the first three exons of the gene as well as 3 kb of upstream promoter sequences. The arrows around exon IV indicate that the exact position of exon IV has not been further determined. Not all EcoRI sites are indicated. (C) ChI is the microinjection fragment that was used to assess the chicken CRABP-I promoter region in transgenic mice. It contains 3.6 kb of chicken CRABP-I sequences, including 3 kb of upstream sequences, exon I, intron I and is linked in exon II to a LacZ reporter gene carrying an internal ribosomal entry site (IRES-LacZ reporter cassette).

To determine the sequence of the gene and the promoter region, fragments spanning the regions indicated in Fig. 1A were subcloned into BluescriptII vectors for sequence analysis. The sequences of the four exons, and deduced amino acid sequence are presented in appendix A. Comparing the coding sequences of the exons of the *Fugu* gene with those of the mouse revealed a 78% sequence homology, and indicated that the intron/exon borders have been conserved. All splice donor and acceptor sites comply with the consensus GT and AG rule (Breathnach, 1981). At the amino acid level the deduced pufferfish CRABP-I protein is 86% homologous to mouse CRABP-I.

Cloning and characterisation of chicken CRABP-I

To clone the genomic chicken CRABP-I locus we used the 310 bp partial chicken CRABP-I cDNA clone ChCRABP C4.5 (Vaessen, 1990) as a probe to screen an EMBL3 SP6/T7 Chicken genomic phage library (Clontech). We isolated a number of clones, none of

which contained the whole gene. Restriction mapping and hybridisation with probes specific for mouse exons I, III and IV showed that phage clone 8 contains the 5' end of the chicken CRABP-I locus, and phage clone 17 contains the 3' end (Fig. 2B). A restriction map of the chicken CRABP-I locus can be drawn from the combination of the two phages as shown in Fig. 2A. Fragments spanning the regions indicated in Fig. 2B were subcloned into BluescriptII. We have determined the sequence of the first three exons and 3 kb of the promoter region. The sequence of these exons, and their deduced amino acid sequence are presented in appendix B. The fourth exon has been located on an 8 kb HindIII fragment, but attempts to sequence it using a mouse exon IV primer were unsuccesful. The sequence of exons I, II and part of III is identical to the partial cDNA sequence found by Vaessen, and differs in two residues from the published partial cDNA isolated by Maden, both of which are located in the PCR primers used to isolate their clone (Vaessen, 1990; Maden, 1992). Comparing the sequence of the first three exons of chicken CRABP-I with that of the mouse reveals an 86% sequence homology, and indicates that the intron/exon borders have been conserved. At the amino acid level the deduced chicken CRABP-I protein is 94% homologous to mouse CRABP-I. The homology between chicken and *Fugu* CRABP-I is 85% at the nucleotide level and 85% at the protein level.

```
                   1
Mouse CRABP-I      MPNFAGTWKM RSSENFDELL KALGVNAMLR KVAVAAASKP HVEIRQDGDQ FYIKTSTTVR TTEINFKVGE GFEEETVDGR
Bovine CRABP-I     .......... .......... .......... .......... .......... .......... .......... ..........
Human CRABP-I      .......... .......... .......... .......... .......... .......... .......... ..........
Chicken CRABP-I    ....R..... .......... .......... .......... .......... .......... .......I.. S.........
Fugu CRABP-I       .......... K......... .....T.... ........N. ........EK .......... ......HI.. E.D.......
Ambystoma CRABP-I                                               ...R .......... ......T... E.N.......
Xenopus CRABP      ....S.H... KQ....E.M. ......L... .I........ A...K.E.ET .......... .......L.G ..D.Q.....
Human CRABP-II     ....S.N..I IR....E... .V....V... .I........ A...K.E..T .......... .......... E...Q.....
Mouse CRABP-II     ....S.N..I IR....E.M. ......M.M. .I........ A...K.EN.T .......... .......I.. E...Q.....

                   81
Mouse CRABP-I      KCRSLPTWEN ENKIHCTQTL LEGDGPKTYW TRELAND-E- LILTFGADDV VCTRIYVRE
Bovine CRABP-I     .......... .......... .......... .......-.- .......... .........
Human CRABP-I      .....A.... .......... .......... .......-.- .......... .........
Chicken CRABP-I    .....A.... ....Y.K... I......... .......-.- ...
Fugu CRABP-I       ..K......S ....R.K... V.......F. ....NG.-.- .T.V...... .........
Ambystoma CRABP-I  ..K..A.... ....Y.K... V..T...... ......E-.- .........
Xenopus CRABP      N.....E... .........V ...E....S. .........A ....MT.... ........L NFWITPSL
Human CRABP-II     P.K..VK..S ...MV.E.K. .K.E....S. ....T..G.- ....MT.... ....V....
Mouse CRABP-II     P.K..VK..S G..MV.E.R. .K.E....S. S...T..G.- ....MT.... ....V....
```

Fig. 3: Sequence alignment of CRABP-I proteins from various species. The deduced amino acid sequences of CRABP-I from chicken and the puffer fish are compared to the known sequences from mouse, rat, cow, axolotl (*Ambystoma*) and human CRABP-I. Mouse CRABP-I is used as the paradigm. Dots indicate identical residues, and amino acid differences are shown.

Analysis of the promoter region of chicken CRABP-I

In addition to the first three exons of the gene we have also sequenced approximately 3.2 kb of promoter sequences of chicken CRABP-I, with the aim of identifying conserved cis-acting elements involved in the transcriptional regulation of the gene. Comparison with the known promoter sequences of the mouse gene did not reveal any homologous elements of significant size. Functional homology between the chicken and mouse promoter regions was assessed with a reporter construct containing chicken CRABP-I promoter sequences (Fig. 2C). The microinjection fragment derived from it, named Ch1, contains 3 kb of upstream sequences, exon I, intron I, and is linked in exon II to a LacZ reporter cassette containing an internal ribosomal entry site (IRES-LacZ cassette) (Mountford, 1994). Three independent transgenic embryos were obtained with Ch1. All three embryos showed specific LacZ staining in the developing hindbrain, in addition to some aspecific staining due to position effects in two of the embryos (Fig. 4D). LacZ expression in the 10.5 day p.c. embryo was restricted to rhombomeres 2, 4, 5 and 6, and at lower levels in 7 and 8, which is identical to a subdomain of the expression pattern of mouse CRABP-I in the hindbrain.

When the similar 3 kb upstream promoter region from the mouse CRABP-I gene is used to drive expression of either a LacZ reporter gene, construct BXhspZ, or an epitope tagged version of CRABP, construct ECTag, expression of the reporter gene is found not only in the hindbrain region, but also in the midbrain, in the cranial nerves and in the mantle layer of the spinal cord (Fig. 4A). This pattern of expression reflects the neuronal subdomain of the endogenous murine CRABP-I expression pattern. The rhombomere specific pattern of CRABP-I expression in the hindbrain of the mouse is illustrated in a section through the head region of an embryo with construct ECTag, showing the expression of the transgene at low levels in rhombomere 2, at higher levels in rhombomeres 4, 5 and 6 and again at lower levels more caudally, in addition to expression in the midbrain and in the spinal cord (Figure 4B). Thus, the staining pattern shown by the chicken promoter transgene appears to form a subdomain of the expression pattern found with similar mouse promoter transgenes, BXhspZ and ECTag (see Materials and Methods).

Analysis of the promoter region of *Fugu* CRABP-I

As with the chicken gene we have also sequenced approximately 5 kb of promoter sequences of the *Fugu* CRABP-I gene, including 3 kb upstream and the complete first intron, to identify conserved regulatory elements. Again we were unable to locate any homologous elements of significant size. Functional homology was assessed by a reporter construct containing *Fugu* promoter sequences (Fig. 1B). The microinjection fragment Fu1 contains 3 kb upstream sequences, as well as exon I and part of intron I, driving a Hsp68-LacZ expression cassette (Kothary, 1989). Three independent transgenic embryos were obtained with Fu1. Interestingly, all three embryos showed specific LacZ staining in rhombomere 4 of the developing hindbrain (Fig. 4E). In the 9.5 day p.c. embryo the rhombomere 4 specific staining was extended to the neural crest cells migrating from rhombomere 4 to the second branchial arch (Fig. 4F). Rhombomere 4 is the site which shows the highest level of CRABP-I expression in the developing hindbrain of 9.5 days p.c. mouse embryos (Maden, 1992). Comparison of the expression pattern of the LacZ transgene driven by *Fugu* promoter sequences with that driven by the mouse and chicken promoters shows that the extent of the expression pattern decreases with

an increase in evolutionary distance between the species from which the promoter was derived and the species in which it was tested, i.e. the mouse.

Discussion

In this paper we present the cloning of the CRABP-I genes from two different species, the chicken and the Japanese pufferfish, *Fugu rubripes*. The *Fugu* gene consists of four exons as is the case for CRABP-I from other species, and as indeed appears to be the case for all other members of the intracellular lipid binding protein family (Bass, 1993). The same is expected of the chicken gene, but we have sequenced only the first three exons. The putative intron/exon borders appear conserved and all contain the expected GT splice donor and AG splice acceptor sites (Breathnach and Chambon, 1981). *Fugu* CRABP-I and chicken CRABP-I are 86% and 94% homologous to the mouse CRABP-I protein respectively. Comparison of chicken with *Fugu* CRABP-I reveals a 85% homology over the first three exons. Taken together with the 100% homology between bovine and mouse CRABP-I, and the 99.3% homology with human CRABP-I, these data show the extremely high degree of conservation of the CRABP-I gene (Fig. 3). This indicates that there has been a strong selective pressure on the gene through evolution. Interestingly this selective pressure has not been confined to the RA binding pocket of CRABP-I but includes the whole protein. This implies that the configuration of the ligand binding pocket is critically dependent on the exact structure of the whole protein, and that a change elsewhere in the protein structure would alter the RA binding capacity significantly, perhaps lowering the affinity for RA below that of the RA receptors. Alternatively CRABP-I may interact with other proteins that require its specific structure for recognition, as has been suggested previously (Donovan, 1995). These interacting proteins could be enzymes involved in RA metabolism or may be the nuclear RA receptors. The presence of CRABP-I in the nucleus of certain cell types has been reported recently, suggesting the possibility of a direct transfer of RA from CRABP-I to the nuclear receptors (Gustafson, 1996).

In addition to the coding regions of the genes we have also sequenced the promoter regions of both the chicken and *Fugu* CRABP-I genes, with the aim of identifying conserved elements that could be involved in the transcriptional regulation of the gene. This approach has proven to be fruitful in the study of the regulation of the Hoxb1 and Hoxb4 genes (Marshall, 1994; Popperl, 1995; Aparicio, 1995; Morrison, 1995). We have investigated the transcriptional regulation of the CRABP-I gene during mouse development (Kleinjan, 1997), and identified a number of elements in the 3 kb upstream region of the gene that direct CRABP-I expression in the developing central nervous system of the mouse. Surprisingly, we are unable to recognise these elements in the 3 kb upstream sequences of either the chicken or *Fugu* genes. This may be due to the fact that the recognition sequences of the transcription factor binding sites have diverged from the consensus sites such that they are no longer recognisable. The expression patterns of mouse CRABP-I and chicken CRABP-I have been well studied (Maden, 1989; Vaessen, 1990; Ruberte, 1991; Maden, 1992; Ruberte, 1992), and their expression patterns during development are largely identical. However, small differences between the two species exist, for instance in the developing hindbrain region. After the appearance of rhombomeres (rh) in the hindbrain, CRABP-I expression in the mouse is found at low levels in rh2, at high levels in rh4-6, and at decreasing levels again caudally (Maden, 1992; Leonard, 1995). In the chicken, CRABP-I is detected at high levels in rh4, at lower levels in rh5, and at high levels again in rh6,

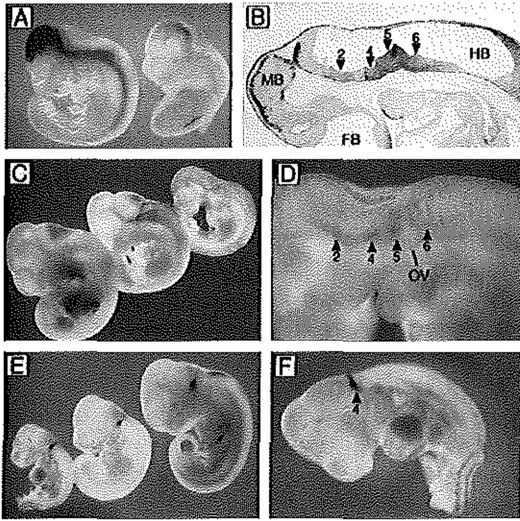


Fig. 4: Transgenic embryos obtained from oocyte injections with a LacZ reporter gene driven by upstream CRABP-I promoter regions from mouse (A, B), chicken (C, D) and puffer fish (E, F). The mouse upstream region drives expression of LacZ in the midbrain, hindbrain and spinal cord. A section through the hindbrain region of a transgenic embryo, carrying an epitope tagged reporter gene (CRABP-Tag) driven by the mouse CRABP-I upstream region, ECTag, shows the specific expression in rhombomeres 2, 4, 5 and 6 of the hindbrain. (C) Transgenic mouse embryos with the chicken promoter region/LacZ construct Ch1, showing expression of LacZ in the hindbrain region. Expression in other sites is due to position effects and therefore non consistent between the embryos. (D) A 9.5 day p.c. Ch1 embryo showing staining in rhombomeres 2, 4 , 5 and 6. (E) Transgenic embryos obtained from injections with the puffer fish promoter region/LacZ construct Fu1. Specific staining is found only in rhombomere 4. (F) In a 9.5 days p.c. embryos the rhombomere 4 staining is extended to the neural crest cells that migrate from this rhombomere to the second branchial arch. FB, forebrain, MB, midbrain, HB, hindbrain, OV, otic vesicle; numbered arrowheads indicate the positions of the rhombomeres.

with a subsequent decrease more caudally (Maden, 1990). At present the expression pattern of CRABP-I in *Fugu* embryos is not known.

We have made transgenic mice with the upstream regions of chicken and *Fugu* CRABP-I driving a LacZ reporter gene, and compared these to the expression patterns generated from either a LacZ or an epitope tagged reporter gene driven by the equivalent mouse promoter region. In three independent transgenic embryos with the chicken promoter construct we find expression of the LacZ reporter gene in the region of the hindbrain that covers rh2 and rh4-6 (Fig. 4B). The restricted expression of LacZ in the hindbrain region suggests that the chicken CRABP-I promoter, when introduced into transgenic mice, drives an expression pattern that only partly recapitulates the endogenous mouse expression pattern. When the promoter region from *Fugu* CRABP-I was tested, the transgenic embryos showed specific and reproducible expression of LacZ only in rh4. Thus the domain of LacZ expression driven by the promoter region of *Fugu* CRABP-I is an even smaller subdomain of the mouse expression domain than that driven by the chicken CRABP-I promoter region.

Thus, the regulatory regions of the gene have been much less conserved than than the coding sequences. The reporter construct containing the *Fugu* upstream region shows that the regulatory mechanisms driving CRABP-I expression have been best conserved in rh4, suggesting that rh4 may be the most important site of CRABP-I expression. Rh4 lies at the boundary between the highly RA sensitive anterior hindbrain and the less RA sensitive posterior hindbrain. RA treatment of early stage embryos results either in complete loss or in a posteriorisation of anterior hindbrain structures. Typically these effects are limited to the hindbrain anterior of rh4.

A respecification of the identity of rh2 into a rh4 identity is seen in mouse embryos treated with RA at day 7.5 p.c. (Marshall, 1992). Interestingly, the restricted expression of the Hoxb1 gene in rh4 is controlled by RA. The expression of Hoxb1 in rh4 is set up by a positive enhancer that generates expression of Hoxb1 which also extends into rh3 and 5. A repressor containing two RA response elements negatively regulates the expression in rh3 and 5 and restricts Hoxb1 expression specifically to rh4 (Studer, 1994). This rhombomere specific activation and repression of Hoxb1 can not be accounted for by a specific distribution of the RARs/RXRs in the hindbrain. CRABP-I is thought to be involved in the regulation of free RA levels. It is well possible that the expression of CRABP-I is specifically important in rh4, where it has to keep the RA that is available for the RARs below a certain treshold level, and that therefore its regulatory mechanisms have been best conserved in that particular rhombomere.

The evolutionary paths of mice and teleost fish, like *Fugu*, have diverged approximately 430 million years ago, while the paths of chicken and mice have diverged 200 million years ago. Thus with increasing evolutionary distance between species an increasing loss of transspecies regulatory potential on the CRABP-I gene occurs. A more detailed analysis of this phenomenon will require the generation of transgenic lines with larger constructs, and the incorporation of more species into the analysis. In this respect it would be very interesting to find out whether amphioxus (*Brachiostoma floridae*), the closest living invertebrate relative of the vertebrates, has a CRABP gene, and whether the promoter region of that gene has any regulatory potential in transgenic mice. Although amphioxus lacks apparent brain structures it has been shown recently that amphioxus embryos treated with RA show patterning defects of their anterior nervous system similar to the those seen in vertebrates (Holland and Holland, 1996).

In summary, we have isolated and sequenced the genomic loci of CRABP-I from the chicken and pufferfish, and confirmed that CRABP-I has been strongly conserved through evolution. The promoter regions from the two species directed the expression of a LacZ reporter gene in transgenic mice to specific subsets of the murine expression domain. A larger part of the murine expression pattern was reproduced by the chicken promoter region than by the pufferfish, reflecting the relative evolutionary distance between the organisms.

Acknowledgements

Filters and clones from the *Fugu* genomic library were provided by C. Burgtorf and H. Lehrach (Berlin). We are thankful to Lien Braam and her staff for animal husbandry, and to Mirko Kuit for expert help with photography. M.J. Vaessen, R. Poelmann and A. Gittenberger-de Groot are acknowledged for their support. D.A.K. and S.D. were supported by a grant from the Dutch Heart Foundation (NWO/NHS grant 900-516-096).

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Appendix A: Genomic sequences from the CRABP-I locus from puffer fish, *Fugu rubripes*. The coding sequences are written in bold face, and the deduced amino acid sequence is indicated underneath in one letter code. The introns have not been fully sequenced and are indicated by a broken line. Two putative poly adenylation signals are underlined.

```
   1  actagtgtga gtcaaaaatc agaacgaatg ttggtgttaa aatgttattc
  51  caatacaagt aaattcataa aatcccagcc ttgaatattg gtccacgctt
 101  acctggtcca gcatccatgg acttaatctg ttttccgagc tcgagatccc
 151  acagacggat gtgtgcatcc agggagctgg aggtcagcga tggctccatt
 201  gtgactgatg tcactgacac tactctcagc tggtgtctca gagtcatgnn
 251  ctgcaggcgc gatgtggggg aaagataccc gacgttcagg gcaaattaga
 301  tcaacattca ggcaaaataa actgctgtag aatatcaaat tctactctta
 351  gtggtgatat taggtaagaa tctggacagt tttggataac tggtgccttt
 401  cacgataaat gactgattat caacagctca cagttactgg ttgtcattac
 451  gacaaagaga tacggtttct tttgaaacaa tgtttactgt ttacattgtt
 501  attggtattt tcagaaaaac caaagccact caccacattt ccagactttc
 551  acatgtcatc taatgaacct gtgatgatag tttctgtccc atctgcctca
 601  cttttacccc acgctgctgt ccagatagca tcatcgtggg ctaagaggga
 651  acacatgcac tcttcatccg tgttctctta atgttagcgc ttcaatacaa
 701  accatgcagt aaaaacaagc aggcctctat atggaaactg aaataaatgt
 751  ttccaaccta ccgtgttctt gcttgaaaag aatgcagtag ctgaaataaa
 801  agaaacagta acagtaaata ttttacccat aaaataggag gacgctcaaa
 851  atgaaagtga agttggttga acttgttgct tacttgagtg ctcattttg
 901  gttctgagag tcacattcag tcagacagct aacctacaca ttaagcgttt
 951  tacattctgc accagctcct tgcaaatgta ttcatgttaa caacatgtta
1001  acacagtagg aatgggataa catttttgag tttaaccaac accattttag
1051  gtttaaccgg tattaatgtg aggagaattt ccacatgagt aatagcattt
1101  tagagatttc tagacattta tgaccagaac aaactgccag tttttaatc
1151  tctttgctgg ttgaagtacc attgtgaaat ataaacttta gcaggagcta
1201  aagtcgactg acagcttagc gctacattga tcacagacaa tgaaacccag
1251  tcagcagatc tagattatcg cctcgtttaa gaagcgaaca atgctgtatg
1301  gtgacgattg tatcatttaa atatatccaa taaatataaa tattgaccca
1351  taccttgaat tcagctagta tggggtttct tcgattaaga gttcccgtgt
1401  attcataagg gagaagccat aacacgacga aacttcggtt tgttcttctt
1451  cgtttctata ccagttaact aataacgcac agagctctac tgccaccagt
1501  ggacaaaaag cgtttgcttc attaggaaaa ttggtaattg agttgtttcg
1551  ctattactgt attacaaaat tattcaatta catttttag gataatatta
1601  acaataatac acactaatca gtgatgattg ttcgtaatca ttctgtttac
1651  attattatgt atttgattat atagtcactg ttatgcagtc tgtcgcagat
1701  agagtgtatg tcaatctatt gcgcgtaatt tatcttacag tcgtgtgcag
1751  aaaaaatgta aatgtatctt tacagtctgt gagcaagtaa agggccgggg
1801  aaccacactg agggtgtggt ttcctgatca ggctgtgatg atgaactgca
1851  acggagggcc cacccaacct gaaaacgggt aaacaagcag acaacaacag
1901  gatcacacaa cgtgaggaat ccctaacaga cagctctgcg acttaagccc
1951  ggcttaaata aagtgtcttg ttaaataatg atcattttaa aaaaaaaatc
2001  cttttgcatg ataagttaag ctaaaaatct gttcttcaga gactgatgtt
2051  ggatatgagg atgcaaaaat ttgtaaaggc ttttaaactg ttatttctat
2101  ttcctagctg aaatcatctg gagatatttt cacaatccaa gtaaaaaatc
2151  cactaaaaag ttcagactca gtctaacttc gctggtgagc ttcagcacac
2201  acatctttca tctattttg taatgctgca ttgtgacaat aaattttaat
2251  aggaggacaa ctttttttta aagttgtgtc tgcaaaaaga atacagttga
2301  tataatcttc cttaatcacc attaaattat atatgtacac tgcaaaaata
2351  tgtaaagtgt gaattacaaa tgtacattgt attctttagt aggactggca
2401  gaatgccaaa cattttgtat tttgcaaaaa taaattacct gccaattttt
2451  tttaatctgt ttttcttatc tgtttctata gaaatgacac aatattgatg
2501  acaatcaatg caaaacctac agttttgccc ccctgtactc atagggcagc
2551  atttttggga ggggtgggtg gccctcctcc cattgagaca gtagctataa
2601  agcgagactg ctggtgtact gagagcgctg tagaggtgag agcgagtgag
2651  gcagaaatat acatttacag gacacccaga aacacacact tgcattttaa
2701  gagtgtgatt ccagagcgga ggacaggagc acagggaagc aggaggagag
2751  gtgagaaaga gatcagagag agcacgaaga cggcagcaag cagtcacaac
2801  tacgcgtgtg gtgagacacg agcgtccttc aagcgacaga gctcctcgcg
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2851  cgctcagacg ctctagacga gaacaactag ATGCCAAACT TTGCCGGCAC
                                        M  P  N   F  A  G  T
2901  CTGGAAGATG AAGAGCAGTG AGAATTTTGA TGAACTTCTC AAAGCCTTGG
        W  K  M   K  S  S   E  N  F  D   E  L  L   K  A  L
2951  gtaagccctc tctcttcctc ctcttgattt ctcacaagca aatcctgtgc

      ------------------ 1.7 kb --- intron I -------------

   1  ttttgttatt gattgaggga agataactga ccgactgggt ctcctgcctc
  51  ctctgtgctg ctcagGGGTC AACACCATGC TGAGGAAGGT GGCCGTGGCG
                      G  V   N  T  M   L  R  K  V   A  V  A
 101  GCCGCCTCCA ACCCGCACGT GGAGATCCGT CAGGACGGGG AGAAGTTCTA
       A  A  S   N  P  H  V   E  I  R   Q  D  G   E  K  F  Y
 151  CATCAAAACC TCCACCACGG TGCGCACCAC CGAGATCAAC TTCCACATCG
        I  K  T   S  T  T   V  R  T  T   E  I  N   F  H  I
 201  GGGAAGAGTT TGACGAGGAG ACGGTGGACG GGAGGAAGTG TAAGgtaacg
      G  E  E  F   D  E  E   T  V  D   G  R  K  C   K
 251  aatcttctcg agcccgatgg aggcaaaact gacaggttgg ggattcttta

      ----------------- 3.2 kb ---- intron II ------------

   1  gatcacattt gctctgtttg ttctgcccac agAGTCTCCC CACCTGGGAG
                                          S  L  P   T  W  E
  51  TCGGAGAACA AGATCCGGTG CAAGCAAACC CTGGTGGAAG GTGACGGCCC
       S  E  N   K  I  R  C   K  Q  T   L  V  E   G  D  G  P
 101  CAAAACCTTC TGGACCCGGG AGCTGAACGG GGACGAGCTC ACACTGgtga
        K  T  F   W  T  R   E  L  N  G   D  E  L   T  L
 151  gctctaaaaa cctgtgacga ctgctctaga gtccagtcac tgggagtgtt

      -------------------- 2.0 kb --- intron III ----------

   1  cttccctctg ttcttccagG TTTTCGGGGC AGACGATGTC GTCTGCACAC
                            V  F  G  A   D  D  V   V  C  T
  51  GAATTTACGT CCGTGAATGA agctctgtgg cctccttcag acaaccgggt
      R  I  Y  V   R  E  *
 101  agaagcccac ctgaccccac cgcgttcaaa cccatcgaca ttctgtctcg
 151  ctaacgtgct ggcgaatatg ccaagtgttc cgtgtgggtc tgcttagtgt
 201  tcgcgtgcag tagaaatgtc tcagtacttc ttaggtgcag gactgacggt
 251  cctggaacca gctgtttatg taggttagtt ccagacgtac tcattagcta
 301  ttcatgtcca gtgacgcgaa ataaaaggag aggagcaaca gtgtctcggc
 351  ttctttattc taaaataaac tgatgttaaa ggcaaagaaa ttgaccctac
 401  tcacctgtcc tgatgtgacc tcagcagatg cctggtctat gagaggtttc
 451  ataccgctgg gtctgaatgt gttccctaac acaccttcag tcaggagctg
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Appendix B: Genomic sequences from the Chicken CRABP-I locus. The sequences encoding the first three exons of the gene are written in bold face, and the deduced amino acid sequence is indicated underneath in one letter code.

```
   1  gaattcctat tgtctcctct ttttatattc taactgtgct atattcattg
  51  tggctcttgt catccacctc tttttctttt tctcttgact ttgtttatgc
 101  ttcttttcat atttttcctt gtctctttct tctcctttaa aaataattga
 151  cctaactgat agatcagagc ttataaaata gaatctcagc ccttactggc
 201  tttaatattc ttattatatg tgtaatcgaa ttggtgtaac tggtacataa
 251  gccatctgaa attattgcaa tttaagttct atctccttct gttgcctgta
 301  tcagttgaag agtggccttg tgtaccaaca aggcggcaca tggggttaca
 351  tcccagtatt tacatacagt ggaaaataga aaagaagaaa agaaccacat
 401  gtttactcac tgaaagttca ctgccttaag gacctggaac gcagagttgt
 451  tctgctttta aacttttctt ggagcaaagt tagattcttt ctgagattgc
 501  agctcaacac agcagacttt ataaatgaat tggtaaatgc agaagaatga
 551  gagggaatat tttgaatgga atgggaaaca taatgattaa aaaaaaaaga
 601  gaagatgcat acaggaagga tggcatggaa gaagtaggtg attaattcaa
 651  ttatttcata tatatgaact tctggttaca aggaaaatat tagctaaaat
 701  agctctgaat atgtgacctg acactagcct gtgctagtag cagcagtgac
 751  cctgagtctc acagaggcat ggttctgtgt gtgcttgatt ttggggtcta
 801  ttctaaagag ctgctgagcc cagtctgcta gcactagaag gaaagcctct
 851  ctctgttgtg ggttaattaa ggtgagaacg catacacact gtcagacaac
 901  accacttaga cacccaagaa tgtctggata ggtggaactg gtgactcaac
 951  aggctttagg caagattata aactaaataa aaatgtagtt tgatttaaat
1001  gtatttgagc tgactagctc aatctgactt ggaccttgca gaggagagcc
1051  tttgccacct gcaggctctt gtttctccta gcttgccaca cttcttaaat
1101  gaagggtatt ctgaagcttc tctcctggtc agcatgaggt aggtcacacc
1151  acagtccaat acctcaaaaa ctggtcctac ctcttaaaaa caccctcaca
1201  gataacgtct ctggaatact tcttggtcag gtcagtggtg cagcaaaaaa
1251  aaaagagcag tagtggaaat taagtgatcc tttaaagacc aaaatgcccc
1301  ccttcaaatc ttcagggcca atttattttc ttaggtagac ataaatggtt
1351  cctacccagt ttctgctgca cttgcttatt atcataatac cttcttggtt
1401  cctgcaggcc caatgagcct gcaccagatc tgtctattgc tacacaaagc
1451  agcgctgaaa ctccctgcag agcgacctca gcttcagctt gccagttaca
1501  ccactgaaag catactgcca cagtccaggc ttgcttccag aaccccacaa
1551  ataaggacac gtggacccca gtggaagcaa tgcagcatct gcaggtggca
1601  aaggctctcc tctgcaaggt cagcagattg agctagtcag ctcaaataca
1651  tttaaatcaa actacatttt tatttagttt ataatcttgc ctaaagactg
1701  tgagtcacca gtccacctat acagtacatt cttgggtgtc taagtggtgt
1751  tgtctgacag tgtgtatgcg ttctcacctt attactcaca gagaggcttt
1801  ccttctagtg ctgcagtaag tcccagatct gcacatttta gacatacctc
1851  aaggaacggg cttgtcgttt cacagagagt gaaacaagtg acgaatggct
1901  gtgcatgtac catggagctg gctcagcaaa gggtttccta tcattaaaat
1951  acatctggct ctgttctgag tgggaaatcc cagccctctg aatcttagag
2001  tatttcctta tgcacaacgg caccagaaat agtccatgtg tcttaaccca
2051  tcaaatcctg ttcctcttaa tcgtgtgggt aaatcagctg caactgagga
2101  acttcagtcc agcacctctg gcctaaaact gggggattcc aagcaaaggc
2151  aatagaaatc gatcatgaaa atgcagcgag gcacagatgg gtgcttcaca
2201  gctgcagcat gccggtatta atttaaaaca gcgggacgag gcttggtgtg
2251  cttctctatg aatcactatg actgcaacgg gccctgctcc ttgggatgca
2301  cgtggaacgc cttgcagccc ccccaagctc tatcctctac ctgcagccga
2351  gcggacgtgt gactacggaa gtgccgccgg gctttgcacg tgccgctgcc
2401  cttcgcctgc tcaaacgggc gggctcggac cgggggcagc cgcgcttcct
2451  cgcccgaccc gcctgccctg gcagccgtgt cccccggcac ccctttggtg
2501  ggggtcgtct gtgtttagcg gtgctgggga tgtgctcacc ccgggctccg
2551  ggtgtgcgcg ggctgtggca cagcctcggg cgccagctgt gatcgtgtgc
2601  aaccgcgcct cggccagcga ggctgagcgg atcgatgact gatccgtgag
2651  cctgagactc tctcacttgt gtttctctcc ttcttccccc ggctgcctct
2701  gggctctgtc atcgagtccc tggtacccag cgcacgttgg acaagtggca
2751  gccgggcccc aggaggaccg cggagcaggg agtgatggat ggatgggggg
2801  ctgcaaggac ggacagacgg aaggctggga gggggaacgg cacgtggcgg
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2851   cgtgcgctgc ttccagggga aggggggcgc cggtgttact ctccccgcgg
2901   tcgcgcgctc actgtgccga gcgagagcga gcagcatcgt ccccgccgcc
2951   cgccccgccA TGCCTAACTT CGCCCGCACC TGGAAGATGA GGAGCAGCGA
                  M  P  N  F   A  R  T   W  K  M   R  S  S  E
3001   GAATTTCGAC GAGCTCCTCA AGGCGCTGGg tgagctcggg acggcgggcg
         N  F  D   E  L  L   K  A  L
3051   ggttggggca acgaaccgat ggaaggggcg ccgggcagcc tgagcagccg
3101   tgccggtccc gggaggctcg gggtgcggca cggggaaggt cctgcgagcc
3151   tactcgggga tccggggcgg gcagggaggg cgcgctctgc tcctcacctg
3201   taagggccgg cggtcctatc cggagcacag ctgccctgct ctgcccggag
3251   tccgtcggct tctcccagca ggggctggca cggctgcctt tccgccccct
3301   gccccgagcc ggactggtgc tgggtgagct tggtgacctc aagctctgct
3351   tcctccccgc ttcgcccagG TGTCAACGCC ATGCTCAGGA AGGTGGCGGT
                           G   V  N  A   M  L  R   K  V  A  V
3401   GGCGGCCGCC TCCAAACCCC ACGTGGAGAT CCGCCAGGAC GGGGACCAGT
         A  A  A   S  K  P   H  V  E  I   R  Q  D   G  D  Q
3451   TCTACATCAA AACTTCCACC ACTGTCCGCA CCACGGAAAT CAACTTCAAA
       F  Y  I  K   T  S  T   T  V  R   T  T  E  I   N  F  K
3501   ATCGGGGAGA GCTTCGAGGA GGAGACGGTG GATGGTCGAA AATGCAGGgt
        I  G  E   S  F  E  E   E  T  V   D  G  R   K  C  R
3551   aagaggagaa ctgtgtgtac gtgtctgttt

       --------------------- 2.5 kb intron II ---------------

   1   atctgtattg cactcctttc ctgcagcagt tagtcttact gctgctcatt
  51   gttgtctctc tgcttcatgc tgctggagaa tataattaat gctattccta
 101   atggttttct tgcctgcagA GTTTGGCCAC CTGGGAGAAT GAAAACAAGA
                             S  L  A  T   W  E  N   E  N  K
 151   TCTATTGCAA ACAAACTCTT ATTGAGGGAG ATGGTCCTAA AACATACTGG
       I  Y  C  K   Q  T  L   I  E  G   D  G  P  K   T  Y  W
 201   ACTCGAGAAT TAGCTAATGA TGAGCTGATT TTGgtaagaa cctctgaccc
        T  R  E   L  A  N  D   E  L  I   L
 251   taccaatgtg tgcaccgtgt taacagcttt ttgttgctat gtagcatggc
 301   ttactacttt tcttaaacct atcctgaaaa actgccattt tcatagttct
 351   ctgcagtaca attcgcacgt caaatctttt cttcttttc ttattttatt
 401   tatttaagaa aaacccacaa atatatcagg ttctcacttg cg
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CHAPTER VII

General discussion

Concluding remarks and future perspectives

Vitamin A and its active metabolites, most notably retinoic acid, exert profound and widespread effects on embryonic development, growth and homeostasis of vertebrate organisms. RA is involved in morphogenetic processes in the embryo, induces differentiation and/or proliferation of many cell lines and tissues, is involved in spermatogenesis and in the maintainance of pregnancy, induces certain cell types to enter into apoptosis, and is required for proper functioning of the immune system. Another vitamin A metabolite, retinal, is the chromophore required for rod vision.

How a simple molecule like retinoic acid can have such a wide spectrum of dramatic effects on many biological processes has been one of the intriguing questions in biology. In recent years the enormous research efforts in the field of retinoid biology have revealed a complex and intricate network consisting of a large number of bioactive RA metabolites, transport proteins, metabolising enzymes, intracellular binding proteins, nuclear receptors and response elements, which together are responsible for the propagation, interpretation and execution of the RA signal. The notion that many feedback mechanisms are active in this network adds another level of complexity. Cross modulation of the RA and the peptide growth factor signalling pathways has been observed, involving interactions between the transcription factors AP-1 and the RXRs.

While the role of a number of players in the RA signalling pathway has become clear over the last few years, the function of the cellular retinoid binding proteins has remained elusive. Two CRABP proteins have been identified in a number of species, designated CRABP-I and CRABP-II. Both bind RA with high affinity, with the RA-affinity of CRABP-I 4 times higher that that of CRABP-II. They function by regulating the availability of RA to the nuclear receptors, but the exact way in which they do so is unclear. A number of functions has been proposed for CRABP-I. These include a role (1) as a cytoplasmic buffer, protecting the cell against excess of RA reaching the nucleus and the nuclear receptors, (2) in the metabolism of RA to polar metabolites, (3) in the translocation of RA into the nucleus or (4) in sequestering RA in those tissues that most critically depend on the ligand under RA deficiency conditions.

Mice lacking functional CRABP-I, CRABP-II and the combination of CRABP-I and -II have been generated. No apparent abnormal phenotype could be detected in these mice, apart from an extra postaxial rudimentary digit in the forepaw of the CRABP-II deficient mice (Fawcett, 1995; Lampron, 1995). As no phenotypic effect was obtained by inactivation of the CRABP-I gene we have taken the opposite approach to obtain insight in the function of CRABP-I in mouse development and homeostasis. Chapter IV describes the generation of transgenic mice with constructs containing large fragments from the CRABP-I locus for the analysis of the transcriptional regulation of the gene during mouse development. Through the choice of an epitope-tagged CRABP-I gene as the reporter in these constructs, micro-injection of these constructs led to the generation of two lines which overexpress CRABP-I within the confines of its own expression domain. The mice from these lines appear phenotypically normal under normal conditions. The effect of RA stress, either excess or deficiency of RA, on these mice will need to be investigated. The lack of an abnormal phenotype in either CRABP null mutant mice or mice with overexpression of CRABP may indicate that the CRABPs are dispensable under normal conditions, but are required to cope succesfully with RA stress situations. CRABP null mutants have been tested under conditions of RA excess (Fawcett, 1995; Lampron, 1995) and deficiency (Mark, personal communication), but no difference was

found between the CRABP-/- mice and wildtype mice. However these conditions are very difficult to control, and thus, if the CRABPs provide only a slight degree of protection against fluctuating RA levels, their influence may go unnoticed under the relatively coarse experimental conditions.

The CRABP proteins have been very highly conserved through evolution. This usually indicates that the protein serves a function which is sufficiently important for the organism to confer a selective advantage in the correct maintainance of the gene. The cloning and sequencing of CRABP-I from chicken and puffer fish further elaborated the notion that strong evolutionary pressure has been exerted on the gene. Thus a paradox concerning the function of CRABP appears to exist, where on the one hand this function appears to be dispensable for normal existence (as exemplified by the knock-out mice), while on the other hand the protein is strongly conserved in evolution. One way to explain the paradox is to make the assumption that the function of the CRABPs is dispensable under laboratory conditions, and thus will not become apparent in the knock-out mice under such conditions, but important in nature where the availability of food and its vitamin A content will be highly variable. It is also conceivable that the presence of CRABP may represent a slight evolutionary advantage, which will only become manifest after a large number of generations. Alternatively, the absence of CRABP may create a disadvantageous behavioral change, which has so far not been detected, or which has no influence under laboratory conditions. As both CRABP-I and CRABP-II are expressed in a specific pattern in the developing nervous system this possibility has to be considered.

The main subject of the research described in this thesis has been the study of the regulation of the CRABP-I gene during murine embryonic development, and is described in chapters III, IV and V. Knowledge of the factors involved in the regulation of the gene would lead to a better understanding of the place of CRABP-I in the RA signal transduction pathway, and possibly to understanding the role of the protein. From the inconclusiveness of the experiments on cell lines, described in Chapter III, it became clear that we needed to study the regulation of the gene in the endogenous situation, i.e. in the mouse embryo. Therefore we have generated transgenic mice with various constructs from the CRABP-I locus. As described in chapter IV the expression pattern of CRABP-I in the developing mouse embryo can be split into two different groups of tissues. Expression in these different tissues is regulated via distinct enhancer elements. Enhancer elements located in the upstream region of the gene are responsible for expression of CRABP-I in a specific domain of the central nervous system (CNS). Expression in neural crest and in mesenchymal tissue is regulated via enhancer element(s) located downstream of the transcriptional start site of the gene. The exact localisation of these elements and the identification of the trans-acting factors that bind to these elements will provide valuable tools for future research towards further understanding of the development of the neural crest and limb bud.

We have concentrated on the elements that direct the expression of CRABP-I in the CNS. In Chapter V the identification and characterisation of these elements is described. Two fragments in the upstream region of the gene appear to be required for expression of the gene in neural tissue. Further analysis of these fragments revealed three enhancer elements on the proximal fragment and one on the distal fragment. The sequence of one of these elements had the hallmarks of a retinoic acid response element (RARE). In a series of bandshift assays, in which specific competitors or antibodies against RARs and RXRs were added, we show that the factor binding this element is indeed a retinoic acid receptor heterodimer. The presence of a RARE in the promoter of the CRABP-I gene had so far gone unnoticed, but is not totally unexpected since

RAREs have been found in many other proteins involved in the retinoic acid signal transduction pathway. The presence of a RARE in the promoter of the CRABP-I gene thus allows for the possibility of modulation of CRABP-I levels in response to its own ligand. However, it still remains to be shown whether the receptors bound to the RARE element do indeed transactivate CRABP-I upon addition of RA. From studies with RA response elements from other promoters it has been inferred that the RAR/RXR heterodimers repress transcription when they are unliganded through binding of a corepressor. Upon ligand binding the corepressor is released, and coactivators can now be bound, resulting in transactivation (Chambon, 1996). Future research will have to determine whether this scenario also applies to the CRABP-I RARE.

Furthermore, it will be interesting to see which particular retinoid is the most active ligand in activation of CRABP-I, and what concentration is needed to achieve a significant level of transactivation. All-trans RA is bound by CRABP itself with high affinity, but its affinity for 9-cis RA is very low. Both all-trans and 9-cis RA are known to induce transcription via RAR/RXR heterodimers, but transcription can also be induced by 9-cis RA liganded RXR homodimers (Zhang, 1992; Mangelsdorf, 1995). It may well be possible that the function of CRABP-I is to shift the balance between the level of all-trans RA to 9-cis RA that is available for the nuclear receptors in the cells in which it is expressed.

If the function of CRABP-I is to provide a buffer against fluctuations in RA concentrations in the RA sensitive tissues of the organism, then the presence of a RARE allows CRABP to respond to changing RA concentrations. The fact that this RARE is not a DR-2 or DR-5 type element, which are the optimal response elements for RAR/RXR heterodimers, but a non-optimal DR-4 element fits into a model in which CRABP-I responds to high concentrations of RA. At low concentrations most RA is bound to CRABP-I in the cytoplasm, and only limited amounts of RA reach the nucleus, where it activates the receptors bound on DR-2 and DR-5 elements in the promoters of target genes. At high concentrations the CRABP in the cytoplasm becomes saturated and a large amount of RA molecules reaches the nucleus. The sub-optimal RARE of the CRABP-I gene is activated and the buffering capacity of the cytoplasm is increased by the synthesis of more CRABP-I. However, CRABP could also serve a role in transporting RA through the cytoplasm and to or into the nucleus, delivering it to the nuclear receptors. In this model the presence of CRABP is responsible for actively creating a difference in the amount of RA becoming available for the RARs between CRABP-I expressing and non-expressing cells. Thus CRABP-I may be functional in augmenting putative RA concentration gradients in the developing embryo. The presence of the RARE in the CRABP-I promoter may be instrumental in increasing the steepness of the gradient by creating a positive feedback loop.

Recently a report appeared in which the same RA response element was identified as a negative regulatory element in the P19 cell line (Wei, 1997). This appears to contrast with our results since we identified the element by screening transgenic mice for positive regulatory elements. The element may however function as both a positive and negative element depending on the cell type, as it is clear that the CRABP-I promoter is differently regulated in cell lines and transgenic mice (this thesis)

Conflicting reports exist on the inducibility of CRABP-I by RA treatment of mouse embryos (Harnish, 1992; Leonard, 1995; Means, 1995). The discrepancy between the various reports may be explained by the importance of the exact timing and dose of the RA treatment, and by the fact that the effects are restricted to specific sites in the embryo. By comparing whole embryo mRNA levels a specific upregulation or anteriorization of CRABP-I in a small part of the embryos is likely to go undetected. Recently it has also been shown that in the EC cell line AB1

CRABP-I is upregulated by RA at low, physiological doses of RA, whereas at higher doses this effect is completely abolished (Chen, 1996). The presence of a DNAse I footprint at this putative CRABP-I RARE element using a nuclear extract from embryonic neural tissue strongly suggests that the expression of CRABP-I in neural tissue during embryonic development is RA dependent. The creation of transgenic mice with reporter constructs carrying mutations in this element, or preferably, by mutating the endogenous element by gene targetting, will have to reveal the true importance of this RARE in the regulation of CRABP-I expression.

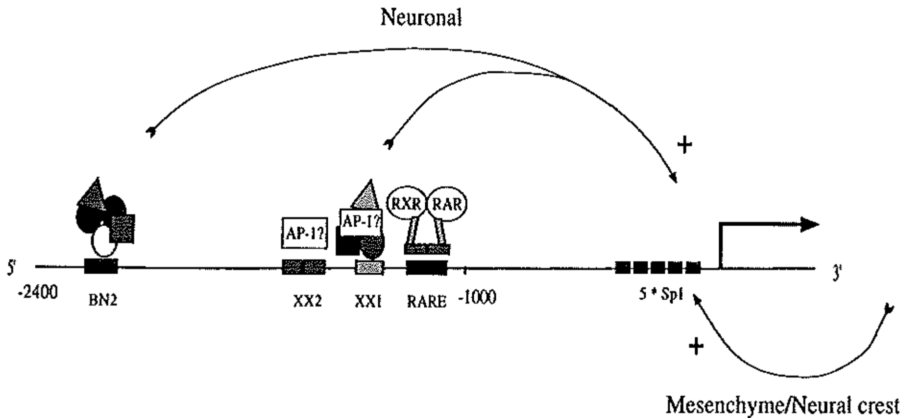

Fig. 1: Schematic representation of the transcriptional regulation of the murine CRABP-I gene during embryonic development. Expression of CRABP-I in its neuronal subdomain is regulated via binding of transcription factors to cis-acting elements located upstream of the gene. Expression of CRABP-I in its mesenchymal/neural crest domain requires the presence of downstream elements. RAR = Retinoic Acid Receptor, RXR = Retinoid X Receptor, AP-1 = Activator Protein-1 (Jun/Fos).

The identity of the factors binding to the other three upstream elements of the CRABP-I promoter is less clear at this stage. Our results suggest that one of the factors binding to two of the elements could be the transcription factor AP-1 (Activator protein I). This factor consists of a dimer of the Fos and Jun proteins, and is known to transduce signals from a number of peptide growth factors. As CRABP-I expression is influenced by peptide growth factors such as certain FGFs (fibroblast growth factor) and BMPs (Bone morphogenetic protein) (Chen, 1996), the presence of AP-1 as one of the components of the complex of factors that regulate neuronal CRABP-I expression would fit into this model. However, it is clear that several other factors are involved in the complex interplay of regulatory factors on the CRABP-I promoter. Some of these are tissue-specific, while others may be more general factors. A schematic representation of the transcriptional regulation of CRABP-I expression is given in figure 1. Further characterisation of these factors may lead to the identification of novel transcription factors, or may link CRABP expression and the RA signal transduction pathway to other known regulatory

pathways, and thus provide new clues to the enigmatic presence of CRABP-I in vertebrate organisms.

As an additional approach to identify the regulatory elements of CRABP-I, we cloned and sequenced the promoter regions of the gene from two other vertebrate species, the chicken and the pufferfish. We expected that the sequence of the regulatory elements, being the binding sites for transcription factors, would be conserved through evolution. However, sequence comparison of the upstream regions with the mouse promoter region did not reveal any significant blocks of homology. Nevertheless, when assayed in transgenic mice, the promoter of the chicken gene directed expression of a reporter gene to a subset of the tissues that show expression with the promoter from the mouse gene. The pattern observed with the pufferfish promoter region in the same assay was even more restricted, essentially to rhombomere 4 only. Thus with increasing evolutionary distance between species an increasing loss of transspecies regulatory potential on the CRABP-I gene occurs. As is argued in chapter VI, the regulatory mechanisms driving CRABP-I expression appear to have been best conserved in rh4, suggesting that this region may be functionally the most important for CRABP-I expression in the developing embryo. In this respect it would be of interest to see whether amphioxus (*Brachiostoma floridae*), the closest living invertebrate relative of the vertebrates, has a CRABP gene, and whether the promoter region of that gene has any regulatory potential in transgenic mice. Although amphioxus lacks apparent brain structures it has been shown recently that amphioxus embryos treated with RA show patterning defects of their anterior nervous system similar to the those seen in vertebrates (Holland, 1996).

In summary, the transcriptional regulation of the CRABP-I gene during mouse embryogenesis requires a complex interplay of many factors. A more detailed analysis of these factors will be very informative not only for a better understanding of the RA signal transduction pathway and the role of CRABP-I therein, but also for a more general understanding of the molecular mechanisms of vertebrate development. The work described in this thesis also provides a basis for further analysis of the conservation of regulatory mechanisms between different species, leading to a broader understanding of transcriptional regulation.

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Summary

Vitamin A (retinol) and its derivatives, collectively referred to as retinoids, exert a wide variety of effects on vertebrate development, cellular differentiation and homeostasis ((Bollag 1996) and references in there). Retinoids, and most notably retinoic acids (RAs) have been of special interest to developmental biologists because of their teratogenic effects on fetal development. Either excess or deficiency of retinoids during pregnancy has been shown to lead to many birth defects. Thus, normal development seems to require a careful balance of retinoids. The mechanisms through which RA affects cellular differentiation and embryonic development involve complex interactions between the products of two distinct gene superfamilies. The first family consists of a group of nuclear receptors for retinoic acid that belong to the superfamily of steroid/thyroid hormone receptors. This family comprises of two groups of RA receptors, the RARs (α,ß,γ), which bind both all-trans RA and 9-cis RA, and the RXRs (α,ß,γ), which have 9-cis RA as their specific ligand. These receptors form heterodimeric complexes and act as ligand controlled transcription factors. The nuclear receptor heterodimers regulate gene transcription through binding to specific DNA sequences, termed RA response elements (RARE), found in the promoter regions of target genes.

The second family consists of serum and cytoplasmic retinoid binding proteins that belong to a superfamily of small proteins involved in the binding, transport, and/or metabolism of low molecular weight hydrophobic molecules. Two members of this family, the cellular retinoic acid binding proteins type I and II (CRABP-I an II) have been shown to interact specifically with RA. Both CRABPs are independently expressed, and bind RA with high, but different, affinities. They have very specific expression patterns in adult tissues and during embryonic development. Their exact physiological role remains to be elucidated, but they are most likely involved in regulating the amount of RA that becomes available to the nuclear receptors.

This thesis describes the studies on the regulatory mechanisms that direct the specific expression pattern of the cellular retinoic acid binding protein type I (CRABP-I) in the developing embryo. Chapter I provides an overview of the effects of RA on embryonic development, of the molecular mechanisms of the RA signal transduction pathway, and gives an introduction to CRABP-I. The production of antibodies against CRABP-I and its family members CRABP-II and CRBP-I (cellular retinol binding protein type I) is described in chapter II. The heart is one of the organs whose development is affected by retinoic acid. To find indications for a putative involvement of the CRABPs in heart development we have performed a detailed study of the expression patterns of the retinoid binding proteins in developing mouse hearts using the antibodies. The study showed that these proteins are differentially expressed during the development of the heart. In chapter III our initial studies on the regulation of CRABP-I using expressing cell lines are described. These studies showed the need to study the regulation of the gene in the endogenous situation, i.e. in the mouse embryo. Chapter IV describes the studies on the regulation of CRABP-I during embryonic development in transgenic mice. By injecting genomic fragments from the murine CRABP-I locus carrying a reporter into mouse oocytes transgenic mice were produced in which the reporter reproduced the endogenous expression pattern of CRABP-I. When different parts were deleted from these fragments and used to produce transgenic mice, the expression pattern of CRABP-I appeared to be composed of two different domains, a neural and a mesenchymal/neural crest domain. Expression in these

domains is regulated via separate regulatory regions. In chapter V a more detailed analysis of the regulatory region driving CRABP expression in the neural subdomain was performed, resulting in the identification of four cis-acting elements. One of these appeared to be a novel RA response element. The transcription factors binding to the other elements could only be partly identified. In addition to the transcriptional regulation chapter IV describes how, using the regulatory regions for both subdomains of the total CRABP-I expression pattern, two transgenic mouse lines were generated with specific overexpression of CRABP-I within its normal expression domain. These lines represent valuable tools for the study of the function of the protein in the development and homeostasis of vertebrate organisms. In chapter VI, the genes for CRABP-I from two other species, the chicken and the puffer fish, were cloned and sequenced. The high degree of homology between different species further substantiates the argument that the protein is extremely well conserved through evolution. The ability of the upstream region of chicken and puffer fish CRABP-I to direct expression of a reporter gene in specific tissues in transgenic mice was studied and a comparison was made with the expression pattern as directed by the mouse CRABP-I upstream region. Interestingly, a larger part of the murine expression pattern was reproduced by the chicken promoter region than by the pufferfish promoter region, reflecting the evolutionary distance between the species.

Samenvatting

Vitamine A (retinol) en afgeleide moleculen, collectief aangeduid als 'retinoiden', oefenen grote invloed uit op de embryonale ontwikkeling van gewervelde dieren, op cellulaire differentiatie en op de algemene homeostase (Bollag 1996). De retinoiden, en met name retinoylzuur, vormen een interessant studie object voor ontwikkelingsbiologen vanwege de teratogene effecten die ze veroorzaken. Zowel een tekort aan retinoiden als een overmaat tijdens de zwangerschap resulteert in een spectrum van congenitale afwijkingen. Normale embryonale ontwikkeling is derhalve afhankelijk van een correcte balans aan retinoiden. De produkten van twee gen-families zijn betrokken bij de moleculaire mechanismen die ten grondslag liggen aan de invloed van retinoylzuur op differentiatie en ontwikkeling. De eerste van die twee gen-families bestaat uit een familie van nucleaire receptoren voor retinoylzuur, die behoort tot de superfamilie van de steroid en thyroid hormoon receptoren. Deze familie bestaat uit twee groepen van retinoylzuur receptoren, de RARs (α,β,γ), die zowel all-trans retinoylzuur als 9-cis retinoylzuur kunnen binden, en de RXRs (α,β,γ), die 9-cis retinoylzuur als specifiek ligand hebben. Deze receptoren vormen heterodimere complexen, en functioneren als ligand afhankelijke transcriptie factoren. De nucleaire receptor dimeren sturen gen-transcriptie door te binden aan specifieke DNA sequenties, retinoylzuur respons elementen genaamd, die aanwezig zijn in de promoter regio van de van retinoylzuur afhankelijke genen.

De tweede familie van genen die betrokken zijn bij de retinoylzuur signaaltransductie route zijn de genen die coderen voor de serum en cytoplasmatische retinoide-bindings eiwitten. Deze behoren tot een familie van kleine eiwitten, betrokken bij de binding, transport en/of metabolisme van kleine hydrofobe moleculen. Twee leden van deze familie, de cellulaire retinoylzuur bindende eiwitten type I en II (CRABP-I and II), binden specifiek en met hoge affiniteit aan retinoylzuur. De beide CRABPs vertonen zeer specifieke expressie patronen zowel in volwassen organismen als tijdens de embryonale ontwikkeling. Ze zijn waarschijnlijk betrokken bij de regulatie van de hoeveelheid retinoylzuur die beschikbaar is voor de retinoylzuur receptoren in de kern van de cel, maar de precieze fysiologe rol van de CRABPs is vooralsnog onbekend.

Dit proefschrift beschrijft de studies naar de regulatiore mechanismen die ten grondslag liggen aan de het specifieke expressie patroon van CRABP-I tijdens de embryonale ontwikkeling. Hoofdstuk I geeft een overzicht van de effecten van retinoiden op embryonale ontwikkeling, en van de moleculaire mechanismen van de retinoylzuur signaal-transductie route. Tevens wordt beschreven wat bekend is over CRABP-I. Hoofdstuk II beschrijft de ontwikkeling van antilichamen tegen CRABP-I en zijn familieleden CRABP-II en CRBP-I (het cellulair retinol bindend eiwit type I). Deze antilichamen zijn vervolgens gebruikt voor een gedetaileerde studie van de expressie patronen van bovengenoemde eiwitten tijdens de ontwikkeling van het hart. Het hart is een van de meest gevoelige organen voor afwijkende retinoylzuur concentraties tijdens haar ontwikkeling. CRABP-I blijkt niet in het hart tot expressie te komen, terwijl CRABP-II en CRBP-I een specifiek expressie patroon vertonen. In Hoofdstuk III is een begin gemaakt met de studie van de regulatie van CRABP-I expressie met een analyse van de promoter regio in twee expresserende cel lijnen. Deze cel lijn studies bleken niet het gewenste resultaat te gaan brengen. Het bleek derhalve noodzakelijk de regulatie van het CRABP-I gen in haar natuurlijke situatie te bestuderen, d.w.z. in het muizen embryo. In hoofdsuk IV wordt de productie van transgene muizen beschreven met constructen die verschillende genomische fragmenten uit het CRABP-I locus bevatten. Deze constructen bevatten het CRABP-I gen, dat

evenwel voor detectie door middel van een moleculaire epitoop gemarkeerd is. De constructen die het gehele CRABP-I locus bevatten bleken in staat het complete expressie patroon van het endogene gen te reproduceren. Productie van transgene muizen met kleinere constructen, waarin bepaalde delen van het CRABP locus waren gedeleteerd, toonden dat het CRABP-I expressiepatroon in het muizen embryo uit (tenminste) twee subdomeinen bestaat, een neuraal domein en een mesenchymaal/neurale lijst domein. De kleinere constructen bleken slechts in een van de twee subdomeinen tot expressie te komen. Een regio aan de 5' kant van het CRABP gen is verantwoordelijk voor de neurale expressie, terwijl de regio aan de 3' kant de expressie in mesenchymaal weefsel en de neurale lijst stuurt.

De neurale enhancer regio van CRABP-I wordt verder geanalyseerd in hoofdstuk V. Dit leidde uiteindelijk tot de identificatie van een viertal DNA sequentie elementen die als bindingsplaatsen fungeren voor de transcriptie factoren die CRABP-I expressie in neuraal weefsel tot stand brengen. Een van deze elementen blijkt een retinoylzuur response element te zijn. In hoofdstuk VI tenslotte wordt de klonering en bepaling van de DNA sequentie van het CRABP-I gen uit twee andere diersoorten, de kip en de puffervis, beschreven. CRABP-I is een evolutionair sterk geconserveerd eiwit, zoals ook uit deze studie blijkt. Hoewel we geen geconserveerde sequenties in de promoter regio's van muize, kippe en puffervis CRABP-I konden identificeren, bleken de promoter regio's van de kip en puffervis in staat om in transgene muizen expressie van een reporter gen te sturen in een bepaald subdomein van het muize CRABP-I domein. De promoter regio van kip CRABP-I bleek een groter subdomein van het muize expressiepatroon te reproduceren dan de puffervis promoter regio, in overeenstemming met de relatieve evolutionaire afstand tussen de soorten.

CURRICULUM VITAE

Dirk Adrianus Kleinjan werd geboren op 15 juni 1968 te Maasland. De middelbare school periode (1980-1986) werd doorgebracht op de Chr. S.G. Johannes Calvijn te Rotterdam en afgesloten met het diploma Gymnasium ß. In datzelfde jaar werd begonnen met de studie Scheikunde aan de Rijksuniversiteit Leiden. De doctoraalstage werd gelopen bij de vakgroep Bioanorganische Chemie (Prof. Canters), gevolgd door twee kortere stages aan Bucknell University, Pennsylvania (USA) en Imperial College, London (UK). Na het behalen van het doctoraal diploma Scheikunde (maart 1992) volgde een aanstelling als onderzoeker-in-opleiding bij de afdeling Celbiologie en Genetica van de Erasmus Universiteit Rotterdam, alwaar het hier gepresenteerde werk werd verricht. Sinds april 1997 is D.A.K. aangesteld als 'post-doctoral scientist' bij de MRC Human Genetics Unit te Edinburgh, Schotland.

List of Publications

M. Ubbink, M.A.G. van Kleef, **D.A. Kleinjan**, C.W.G. Hoitink, F. Huitema, J.J. Beintema, J.A. Duine and G.W. Canters **(1991)** Cloning, sequencing, and expression studies of the genes encoding Amicyanin and the ß-subunit of Methylamine dehydrogenase from *Thiobacillus versutus.* Eur. J. Biochem. 202, 1003-1012.

Miller, A.D., Maghlaoui, K., Albanese, G., **Kleinjan, D.A.**, Smith, C. **(1993)**. *E.coli* chaperonins cpn60 (GroEL) and cpn10 (GroES) do not catalyze the refolding of mitochondrial malate dehydrogenase. Biochemical Journal 291 (1):139-144.

Kleinjan, D.A., Dekker, S., Vaessen, M.J., Grosveld, F.G. **(1997)** Regulation of the CRABP-I gene during mouse embryogenesis. Mechanisms of Development **67**, 157 - 169.

Kleinjan, D.A., Dekker, S., Guy, J.A., and Grosveld, F.G. **(1998)** Cloning and sequencing of the CRABP-I locus from the chicken and pufferfish; Analysis of the promoter regions in transgenic mice. Transgenic Research, in press.

Acknowledgements

De tot stand koming van dit proefschrift is mede te danken aan de samenwerking met, en medewerking van een groot aantal mensen. Een ieder die in de afgelopen jaren op de een of andere manier een bijdrage heeft geleverd wordt hiervoor hartelijk bedankt.

Een klein aantal van deze personen kan er niet aan ontkomen om met name genoemd te worden: Frank Grosveld, voor alle hulp, technische en tactische adviezen en vooral voor zijn inspirerende voorbeeld; Sylvia Dekker, voor al haar werk aan het CRABP project en voor het willen optreden als paranimf; Marie-Josee Vaessen en Arthur van der Kamp, voor de begeleiding, met name in de beginfase van het project; Frits Hali, als mijn tweede paranimf, An Langeveld en Sylvia Dekker, als compagnons tijdens ontelbare lunch- en koffiepauzes; Mijn ouders, voor alle hulp (altijd en overal); en Jacky Guy, niet alleen voor haar 'editorial help', maar voor nog veel meer.

10. Uit het feit dat oogontwikkeling zowel in vertebraten als in *Drosophila* afhankelijk is van de functie van PAX6 kan niet zonder meer de conclusie worden getrokken dat de ontwikkeling van het oog in deze soorten een gezamelijke evolutionaire oorsprong heeft.
    Quiring et al. (1994), Science 265, 785-789.
    Halder et al. (1995), Science 267, 1788-1792.

11. Misexpressie van PAX6 in *Drosophila* eyeless mutanten kan de vraag beantwoorden of de ontwikkeling van ectopische ogen gebaseerd is op auto-inductie van het PAX6/eyeless gen.

12. De correcte vouwing van een eiwit wordt niet in alle gevallen uitsluitend door de aminozuurvolgorde bepaald.
    Shinde et al. (1997) Nature 389: 520.

13. De invoering van de spelregel bij het voetbal dat de keeper een teruggespeelde bal niet met de handen mag pakken heeft duidelijk gemaakt waarom veel keepers keeper zijn.

14. Een verkeerd gedraaid telefoonnummer is nooit in gesprek.

15. Roken is zelfmoord plegen in slow-motion.

16. Op de verpakkingen van produkten betekent het woord 'gesorteerd' meestal 'zomaar willekeurig wat bij elkaar gestopt'.

17. Beter geen weer dan helemaal geen weer.

Edinburgh, december 1997.
D.A. Kleinjan.