ELSEVIER

Mechanisms of Development 67 (1997) 157–169

Regulation of the CRABP-I gene during mouse embryogenesis

Dirk A. Kleinjan[1], Sylvia Dekker, Marie-Josée Vaessen, Frank Grosveld*

Department of Cell Biology and Genetics, Erasmus University Rotterdam, PO Box 1738, NL-3000 DR Rotterdam, The Netherlands

Received 9 June 1997; received in revised form 23 July 1997; accepted 24 July 1997

Abstract

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 regulation of CRABP-I expression in the mouse embryo. We show here that a 16 kb fragment harbours all the elements needed for the 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. Two distinct fragments in the upstream region are required for expression in the CNS, as neither of these fragments alone is able to drive correct expression of a reporter gene in transgenic mice. DNAseI footprinting analysis of the two upstream fragments revealed the presence of a number of protected elements. One of these regulatory elements has the hallmarks of an RA response element, suggesting that CRABP-I expression in neural tissue can be directly modulated by RA via the RARs/RXRs. © 1997 Elsevier Science Ireland Ltd.

Keywords: CRABP-I; Transcriptional regulation; RARE; Transgenic mice

1. Introduction

Retinoic acid (RA), a naturally occurring metabolite of vitamin A, plays a vital role in normal physiology of vertebrates. Maintenance 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. Under normal conditions RA is believed to be involved in the anterior-posterior patterning of the embryo, including the central nervous system (CNS) (Means and Gudas, 1995).

The profound effects of RA on biological systems are mediated through two classes of proteins, i.e. 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 et al., 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 et al., 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 et al., 1994; Napoli et al., 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 et al., 1991, 1992; Lyn and Giguere, 1994; Horton and Maden, 1995). In the limb bud a graded

* Corresponding author. Tel.: +31 10 4087159; fax: +31 10 4360225.

[1] Present address: MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, UK.

0925-4773/97/$17.00
PII S0925-4773(97)00116-0

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 et al., 1988; Perez-Castro et al., 1989), but the presence of an A/P gradient was not found by others (Dolle et al., 1989; Ruberte et al., 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 neural tube. Interestingly these sites of expression coincide with the structures that are most sensitive to RA excess (Vaessen et al., 1990). Both CRABP-I and CRABP-I/CRABP-II deficient mice have been generated (de Bruijn et al., 1994; Gorry et al., 1994; Lampron et al., 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 et al., 1995). Overexpression of the protein in F9 cells (Boylan and Gudas, 1991) and ectopic expression of the protein under control of a heterologous promoter in transgenic mice (Wei and Chen, 1991; Perez-Castro et al., 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 et al., 1994). Thus, while the knock-out of CRABP-I did not reveal a function, overexpression of the protein may give some indication of its role in embryonic development. Knowledge of the regulatory elements would open the possibility of overexpressing 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, i.e. 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. The enhancer region that directs the expression of CRABP-I in the neural domain was studied further, leading to the identification of four regulatory elements. One of these elements is a putative RA response element, which would allow for modulation of CRABP-I expression by RA. The other three elements are apparently able to bind multiple factors, suggesting that a complex interplay of transcription factors regulates the expression of CRABP-I during murine embryonic development.

2. 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 et al., 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.

2.1. 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 is located upstream of the CRABP-I start site. Five independent lines were obtained that were transgenic for this construct. Two out of the 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 in the mantle layer of the neural tube. In a 10.5 d.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 et al., 1992; Leonard et al., 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 neural tube and the neural crest derived dorsal root ganglia are also positive for CRABP-Tag (Fig. 3A,B). In the limb buds of 10.5 d.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

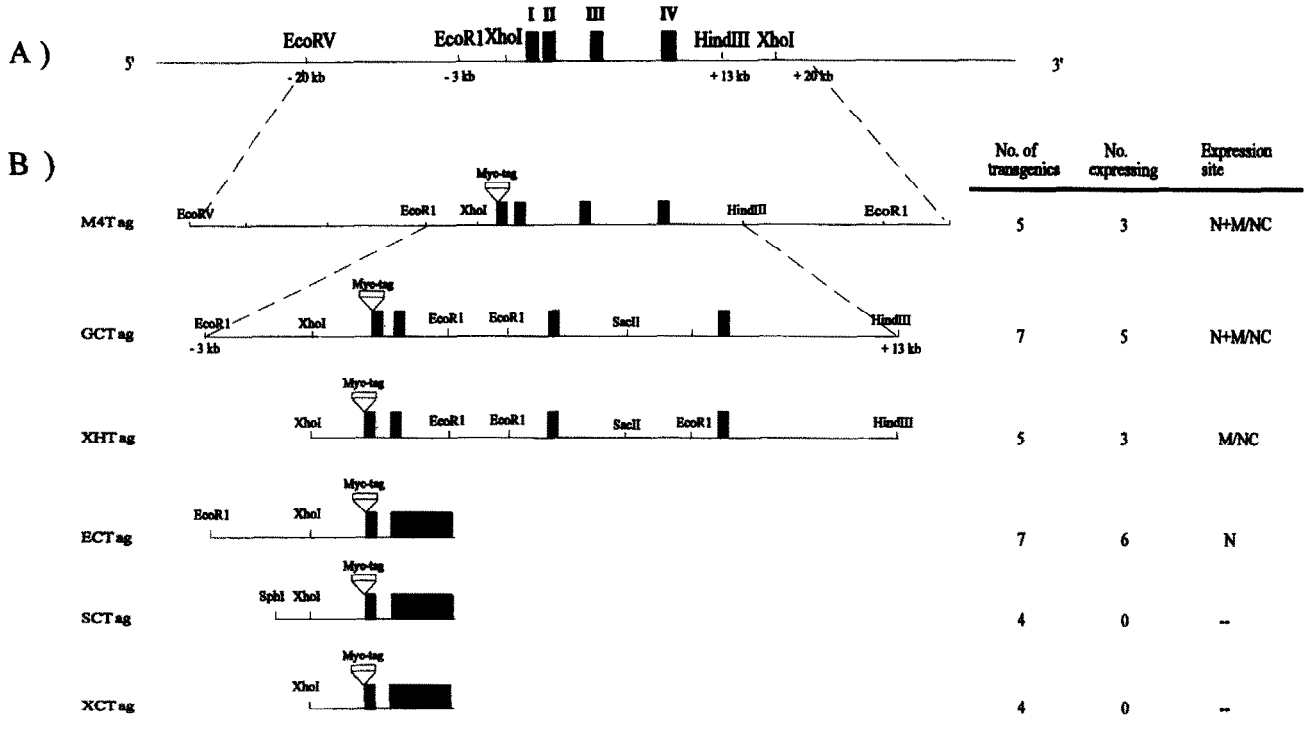


Fig. 1. (A) Genomic locus of murine CRABP-I. Black boxes indicate the four exons of the gene. (B) Fragments used to generate transgenic mice. All fragments contain the complete CRABP-I coding sequences, into which an epitope tag, derived from human c-myc, has been inserted. M4Tag contains 20 kb upstream sequences and 20 kb downstream sequences. The 16 kb fragment GCTag contains 3.2 kb upstream sequences and 13 kb downstream sequences. XHTag has 1.1 kb upstream sequences and 13 kb downstream sequences. 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 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 shows the number of transgenic mice, the second column shows the number of mice that express the transgene and the third column shows 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.

axis. At 13.5 days CRABP-Tag protein is found in the proximal interdigital region in the cells surrounding the cartilaginous condensations (data not shown). In addition to this we find expression in the otic vesicle (Fig. 2B,D). This 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.

2.2. 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 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 neural tube, i.e. the neural tissues that express CRABP-I (Figs. 2H,I and 3E,F). None showed expression in any of the other CRABP-I expression sites. None of eight 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 were 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.

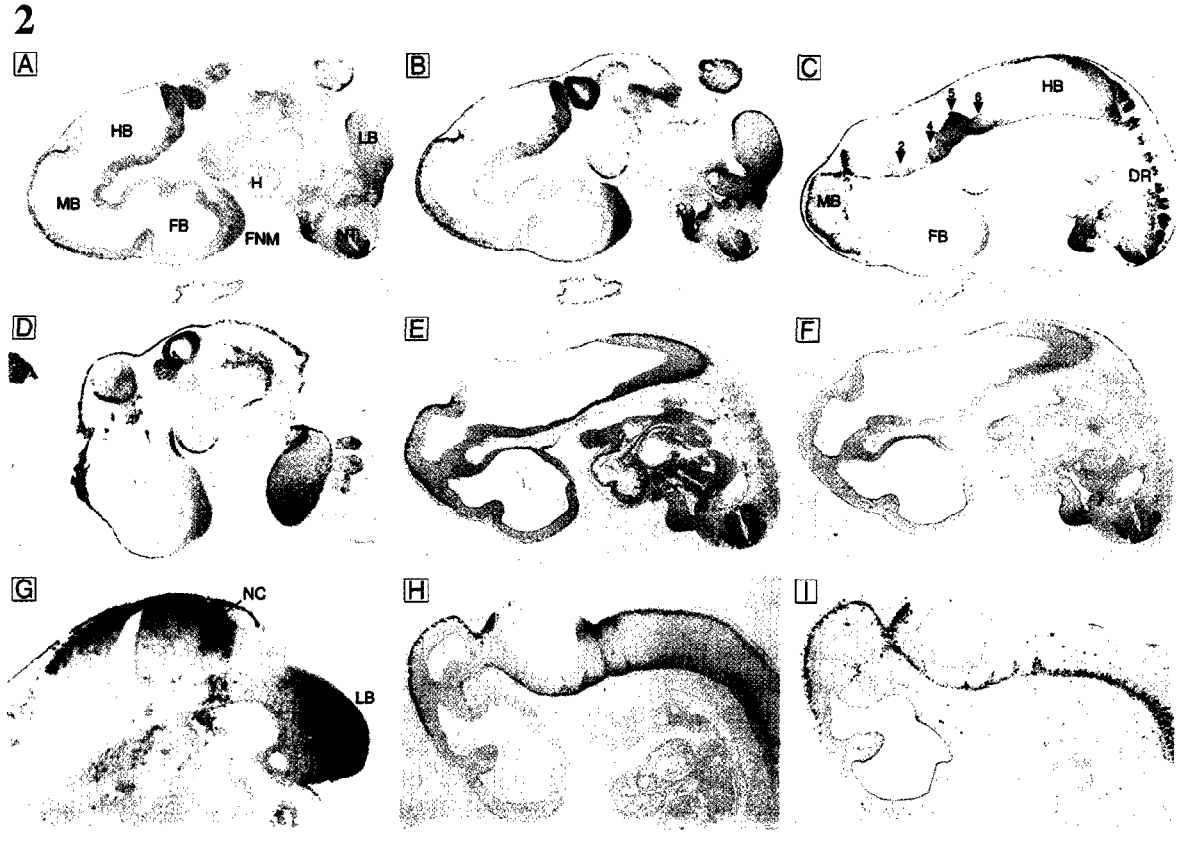
The construct XHTag was designed to test whether intragenic or 3′ sequences are involved in regulation of CRABP-I expression. It starts at the XhoI site 1 kb upstream of the CRABP-I gene and ends at the HindIII site 2 kb after the fourth exon. In comparison to GCTag it lacks 2 kb of 5′ sequences, but 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 neural tube (Figs. 2E–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).

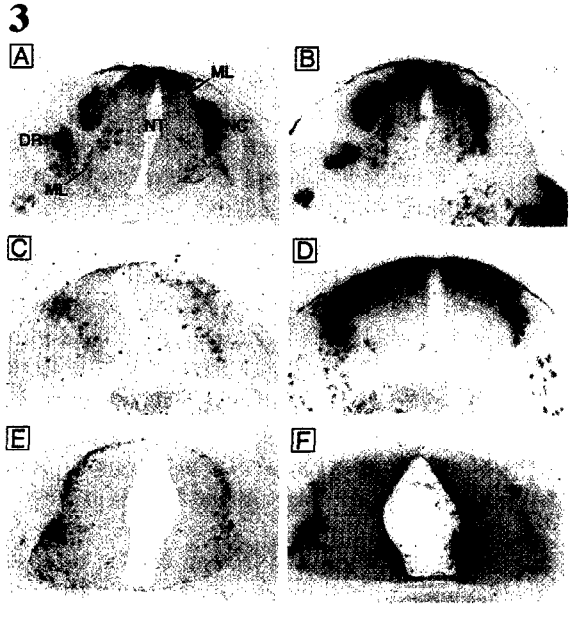
The pattern seen with GCTag can be reconstructed by overlaying the expression patterns of ECTag and XHTag.

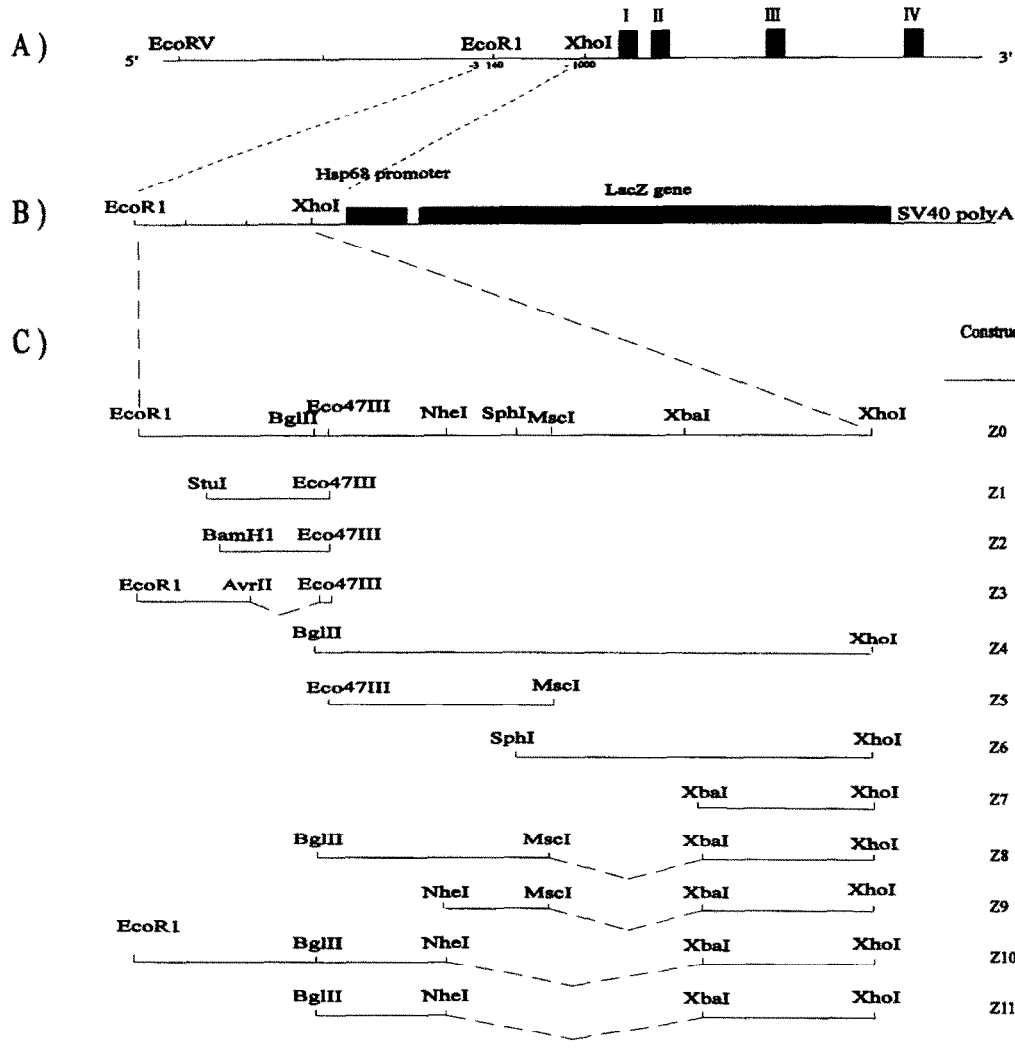
This shows that the CRABP-I gene is regulated via at least two independent enhancer regions, one region responsible for the neural component of its expression, located upstream of the gene and one responsible for its mesenchymal/neural crest component, located downstream from exon II.

2.3. Deletional analysis of the neural enhancer fragment

RA has profound effects on the morphogenesis of the central nervous system. The restricted expression pattern of CRABP-I suggests it may be involved in patterning the CNS. We wanted to know whether the CRABP upstream elements could also act as independent enhancers. Therefore, we cloned the 2 kb EcoRI/XhoI upstream fragment onto a heat shock promoter (hsp68)/LacZ gene, resulting in construct EXhspZ (Z0, Fig. 4). The hsp68/LacZ construct does not give any constitutive expression in transgenic mouse embryos, making it a useful vector for testing the







Construct	No. of transgenics	No. of blue staining *	No. CRABP-I pattern
Z0			
Z1	7	6	neural band
Z2	6	4	neural band
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	3	3	3

Fig. 4. 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. The table on the right shows the expression in transgenic mice. The first column indicates the name of the construct, the second column gives the total number of transgenic mice with that construct, the third column indicates the number of LacZ expressing mice and the fourth column shows the number of mice that expressed LacZ in the neural subdomain of the CRABP-I expression pattern. neural band, band of X-gal staining cells in the bottom third of the neural tube of transgenic embryos.

Fig. 2. Immunohistochemical analysis of GCTag, XHTag and ECTag transgenic mouse embryos at 10.5 d.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–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, neural tube, dorsal root ganglia, limb bud and frontonasal mesenchyme (A–D). Rhombomere specific staining is seen at a low level in rhombomere 2 and at a 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–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.

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

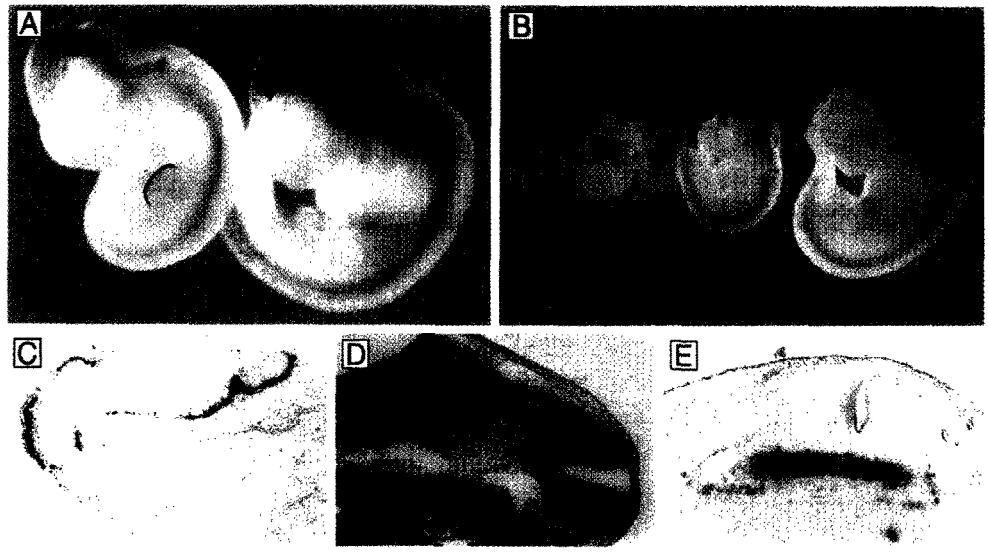


Fig. 5. X-gal staining of LacZ transgenic embryos. (A,B) Transgenic embryos for construct Z4 show LacZ expression in the midbrain, hindbrain, cranial nerves and neural tube. (C) Cross section of an X-gal stained Z4 embryo to indicate the staining pattern in the outer layer of the midbrain and hindbrain. (D) Section of a transgenic embryo showing X-gal staining in the mantle layer of the neural tube. (E) Cross section of the neural tube 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.

presence of regulatory elements in heterologous sequences (Kothary et al., 1989). A series of deletion constructs was made from Z0 (Z1–Z11) and the results of these transgenic experiments are compiled in Fig. 4. Z4 was expressed in the neural CRABP-I expressing cells in the midbrain, hindbrain and neural tube (Fig. 5), consistent with the pattern found for ECTag. Within the Z4 fragment the sequences between BglII-NheI and XbaI-XhoI are found to be important for the expression of LacZ in neural tissue. Both of these smaller fragments are 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 in ectopic tissues due to a position effect. Interestingly Z1 and Z2 show consistent but aberrant expression of the LacZ reporter gene in a specific band in the neural tube (Fig. 5E). The significance of this for regulation of the endogenous CRABP-I gene is unclear, as this is not a site of CRABP-I expression. It may be an artefact created by placing the fragment in an unnatural environment.

2.4. 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, DNAseI footprinting analysis was performed with nuclear extracts, made from dissected midbrain, hindbrain and neural tube tissue from approximately 120 11.5-day-old mouse embryos (Fig. 6). Three distinct protected regions were seen in the XbaI-XhoI fragment (XX1, XX2 and RARE) and one region in the BglII/NheI fragment (BN2). The RARE area contains a direct repeat (DR) with half-site sequences closely matching the AGGTCA consensus sequence found in other nuclear hormone receptor response elements (Leid et al., 1992). The motif is AGGTCCTTAAAGGTCA (in reverse orientation) and has a spacing of 4 bp between the half-sites (DR-4), which is normally associated with binding of a thyroid hormone (TR)/retinoid X receptor (RXR) heterodimer (Umesomo et al., 1991), but can also be a response element for RAR/RXR heterodimers (Leid et al., 1993; Mader et al., 1993). Alternatively, it could bind 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 non-radioactive oligo (self) abolished these retarded complexes (Figs. 7 and 8).

We first tested an oligo RARE, encompassing the putative RA response element, in bandshift assays. Competition experiments with radioactively labelled oligo RARE and excess of unlabelled mutated oligonucleotides were carried out. The mutation of one of the half-sites in such a way that the putative NGFI-B binding site is lost, but a new palindromic repeat is generated (oligo RAREMut) has very little effect on binding of the factor(s) to this oligo. Changing the spacing between the half-sites (oligo RARE7) from 4 to 7 resulted in only 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 RAREΔ2) the ability to compete was lost completely. Competition with the DR-5 RARE from the RARβ gene was also effective, indicating that the factor

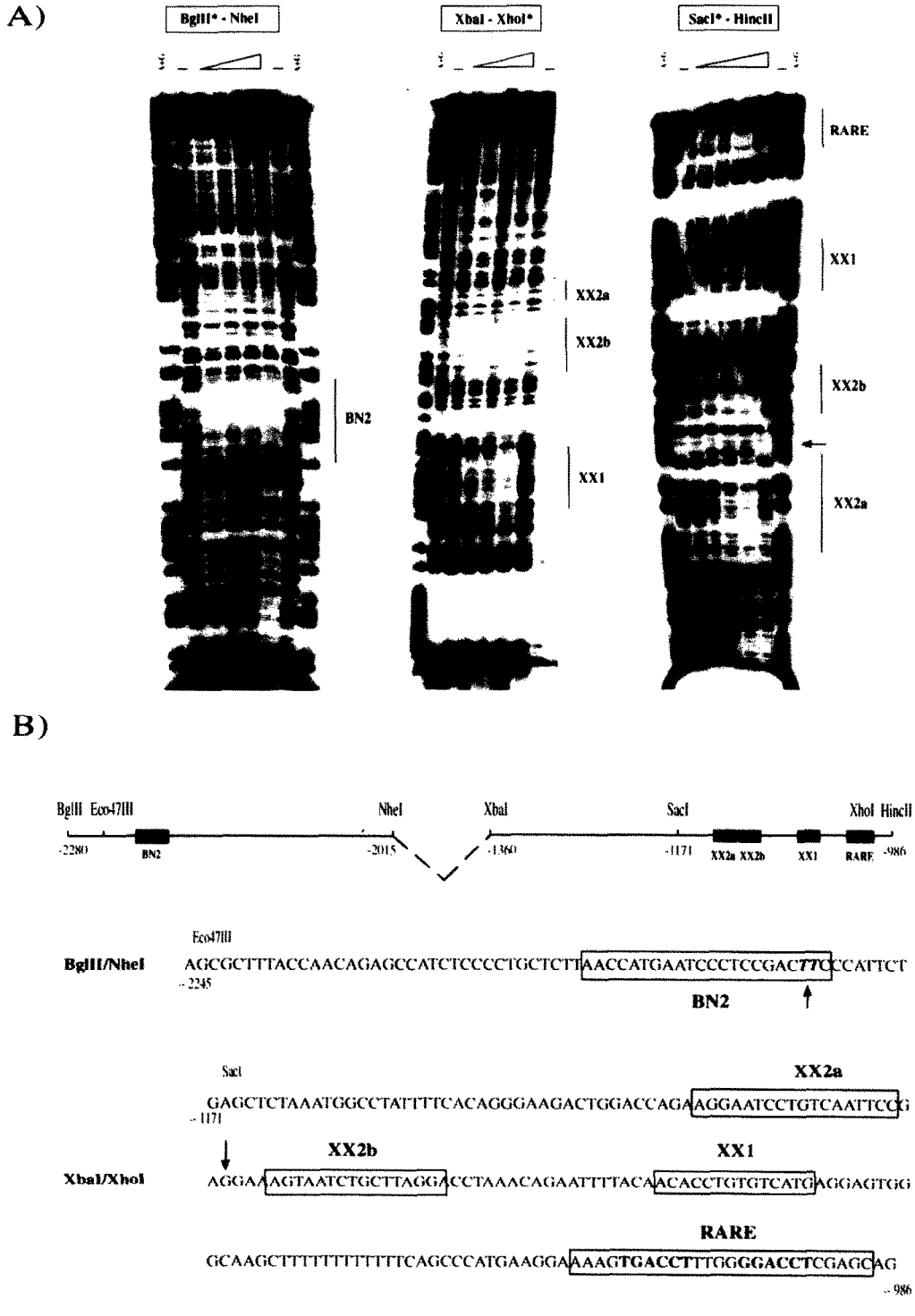


Fig. 6. DNAseI footprinting analysis of the CRABP-I neural enhancer region. (A) The BglII/NheI and XbaI/XhoI fragments required for CRABP-I expression in neural tissue were subjected to DNAseI footprinting analysis. The BglII/NheI fragment spans the region from −2280 to −2015 and the XbaI/XhoI fragment spans the region from −1360 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 labelled end marked by an asterisk. The labelled fragments were incubated with 5, 10, 20 or 40 μg of nuclear extract prepared from excised neural tubes, midbrains and hindbrains from 11.5 d.p.c. embryos before digestion with DNAseI. Only DNAseI (no extract) treated 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.

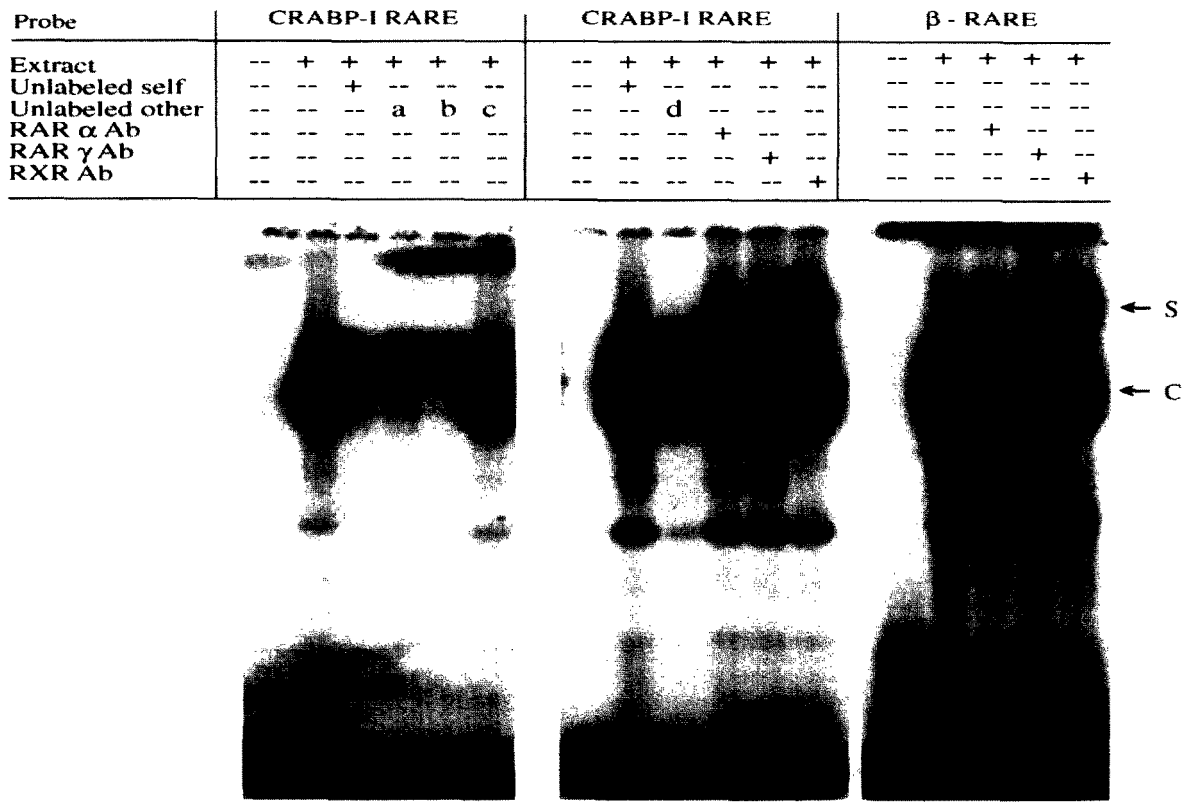


Fig. 7. Bandshift assay on the putative CRABP-I retinoic acid response element (RARE). A labelled double stranded oligonucleotide from the wild-type CRABP-I RARE element was incubated with 3 μg of embryonic neural nuclear extract in the presence or absence of various mutated oligonucleotides (a–c) or the RARE element from the RARβ2 promoter (d). Competitor a is oligo RARE7, competitor b is oligo REREMut, competitor c is oligo RAREΔ2 and competitor d is oligo β-RARE. Addition of antibodies against RARα, RARγ1 and RXR (α, β, γ) resulted in a supershift of the retarded complexes. The panel with the labelled RARβ2 RARE oligonucleotide as probe (βRARE) 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. The specific retarded complexes are indicated by C, while the supershifted complexes are indicated by S.

binding to the CRABP-I response element could be a RAR/RXR heterodimer. We tested this hypothesis by adding antibodies against RARα, RARγ1 and RXR (all RXRs) in the bandshift and found that the antibodies did indeed supershift the complex formed on the RARE element (Fig. 7). The weaker supershift observed with the RARα and RARγ1 antibodies compared to the RXR antibody is likely to be due to a lower titer of these antibodies or could possibly indicate that RARβ is involved in the complex binding to the element.

Bandshift assays with the BN2, XX1 and XX2 elements showed the formation of three to five complexes on each of these elements (Fig. 8). Competition experiments were also performed on these elements. We designed three systematically mutated oligonucleotides (oligos XX1.1, XX1.2 and XX1.3). The mutation in XX1.3 did not interfere with its ability to compete with labelled oligo XX1. Oligo XX1.2 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 XX1.1 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. 8). Since this competition is also lost in the experiment with oligo XX1.2 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 is added as a competitor. The identity of the 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 et al., 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 observation that an oligo, containing the published sequence

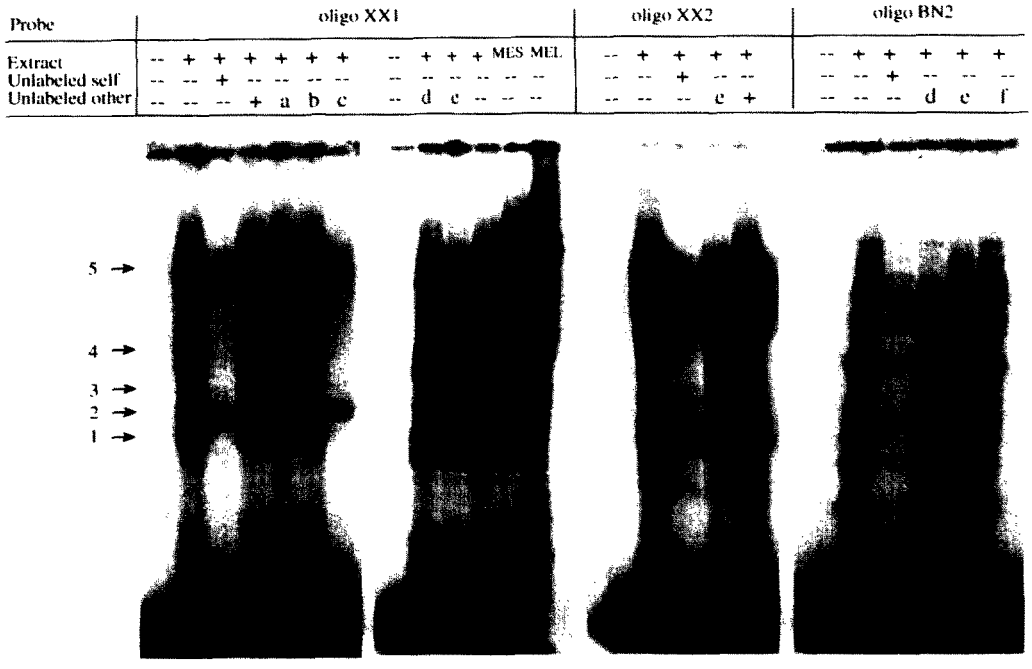


Fig. 8. Bandshift assays with oligonucleotides encompassing the sequences that were found to be protected in the DNAseI footprinting assay. The radioactively labelled oligonucleotide used as probe in each experiment is indicated above the gel. A 100-fold excess of the following unlabeled competitors was used in the lanes indicated: a, oligo XX1.1; b, oligo XX1.2; c, oligo XX1.3; d, oligo 2*Sp1; e, oligo 2*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 a competitor.

plus an additional mutation, did not give a bandshift and was unable to compete with BN2 (Fig. 8).

3. 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 neural tube, 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.

In the limb bud we observed a proximo/distal gradient of the transgene with the highest expression levels found distally, consistent with the expression observed by others (Dolle et al., 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 et al., 1988; Perez-Castro et al., 1989), while the absence of a gradient has been reported by others (Dolle et al., 1989; Ruberte et al., 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 the 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 controlling 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.

3.1. 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 neural tube. 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. We show that this region can act as an independent enhancer on heterologous promoters since it is also able to drive LacZ expression in the same neural tissue when cloned into an hsp68LacZ vector (Z4, Fig. 4). A more precise definition of the cis-acting elements through injection of a series of deletion mutants (Z5–Z11, Fig. 4) showed a requirement for the presence of two fragments of 270 and 350 bp.

The constructs Z1 and Z2 which contain sequences further upstream of the CRABP-I promoter region also drove expression of the LacZ reporter gene in transgenic embryos. However, the LacZ expression with these constructs was consistently found in a particular band in the bottom third of the neural tube, throughout its thickness (Fig. 5E). No endogenous CRABP-I expression is detected at that particular site in the neural tube. Although it is reminiscent of CRABP-I expression found at later developmental stages in the commissural neurons of the neural tube (Maden et al., 1992; Ruberte et al., 1992), we presently believe it to be an artefact caused by taking the element out of its normal environment, resulting in the ectopic activation of the element.

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

DNAseI footprinting analysis of the two upstream fragments revealed four protected regions that we termed BN2, XX1, XX2 and RARE. Bandshift assays with oligonucleotides encompassing the protected sequences 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 et al., 1992) consisting of a direct repeat (DR) with a spacing of four nucleotides (DR-4). A DR-4 element is usually indicative for binding of thyroid hormone receptor/retinoid X receptor heterodimers (TR/RXR) (Umesomo et al., 1991), but depending on the context of the repeat, binding of RAR/RXR heterodimers to certain DR4 elements has also been found (Nagpal et al., 1992; Mader et al., 1993). A DR-4 element is part of a complex response element in the laminin B1 gene, which is induced by RA in F9 teratocarcinoma cells (Vasios et al., 1989). We have used antibodies against RARα, RARγ1 and RXR (all isoforms) to show that the complex binding to the 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. However, it is 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 RA treatment, when the RA was administered within a particular developmental time period (7–8.5 d.p.c.) (Leonard et al., 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 et al., 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 and Gudas, 1996). This may explain why in some studies no upregulation of CRABP-I was detected after treatment of embryos with an excess of RA (Harnish et al., 1992).

One of the factors binding to the elements XX1 and XX2 could be AP-1 or an AP-1-like factor, as an oligo containing two AP-1 consensus sites largely competed with 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 remain unidentified. The elements do not contain recognisable consensus sequences for binding sites of known transcription factors. Thus, the sequence of these 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 (Fig. 8).

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 and Chang, 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. Bandshift experiments with oligonucleotide XX1 have shown that specific retarded complexes that are formed with the nuclear extract from excised neural tissue are absent when MES-1 or MEL cell nuclear extract is used, indicating that the element XX1 binds different regulatory factors in different tissues or cell lines. In addition it is likely that the local chromatin environment plays an important role in the spatiotemporal specific regulation of the CRABP-I gene and this factor is clearly not taken into account in transient transfection assays.

In summary, we show 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 putative RA response element with a 4 bp 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.

4. Experimental procedures

4.1. Constructs

A 5500 bp 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 et al., 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.

The LacZ reporter constructs were made by cloning the fragments EcoR1-Eco47III, Eco47III-MscI and EcoR1-XhoI onto the hsp68 minimal promoter, the LacZ gene and the SV40 polyadenylation signal (Kothary et al., 1989). This resulted in the constructs EE47hspZ, E47MhspZ (Z5) and EXhspZ (Z0), respectively. Microinjection constructs Z1 and Z2 were made from EE47hspZ. Construct ΔABhspZ (Z3) was made by deleting an AvrII/BglII fragment from EE47hspZ. Z4, Z6 and Z7 were made from EXhspZ (Z0). Construct ΔMXhspZ (Z8) was made by deleting an MscI/XbaI fragment from EXhspZ. Both Z8 and Z9 were derived from this construct. Deletion of an NheI/XbaI fragment from EXhspZ (Z0) created ΔNXhspZ (Z10). The fragments Z10 and Z11 were made from this construct.

4.2. Transgenic mice production and processing of the embryos

Microinjection was performed according to standard procedures (Hogan et al., 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 h in 35% methanol, 35% acetone and 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) and 0.02% Nonidet P-40 (NP-40).

4.3. 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/Tween-20 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% Tween-20. 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 3 h. 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 $K_4Fe(CN)_6 \cdot 3H_2O$, 2 mM $MgCl_2$, 0.01% sodiumdeoxycholate, 0.02% NP40 and 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal).

4.4. DNAseI footprinting analysis

The midbrain, hindbrain and neural tube were excised from approximately 120 embryos at 11.5 d.p.c. Crude nuclear extracts were prepared from the excised tissue as described (Andrews and Faller, 1991). 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 footprinted as described (de Boer et al., 1988). Gels were dried and exposed using a PhosphorImager (Molecular Dynamics).

4.5. Electrophoresis mobility shift assay

Oligonucleotides (Eurogentec) corresponding to the DNAseI footprints were analysed by EMSA as described (de Boer et al., 1988). The following oligonucleotides were used as probes or competitors (coding strand sequences are listed; in competitor oligos mutated nucleotides are indicated by lower case letters): oligo XX1, GAATTTTACAACACCTGTGTCATGAGGAGTG; oligo XX1.1, GAATTggcaccaACCTGTGTCATGAGGAGT; oligo XX1.2, GAATTTTACAACAaagtttgCATGAGGAGT; oligo XX1.3, GAATTTTACAACACCTGTGTacgtcttAGT; oligo RARE, AGGAAAAGTGACCTTTGGGGACCTCGAGCA; oligo RARE7, AGGAAAAGTGACCTTTGGttcGGACCTCGAGCA; oligo REMut, AGGAAAAGgtcaagTTGGGGACCTCGAGCA; oligo RAREΔ2, AGGAAAAGctgaagTTGGtacagaCGAGCA; oligo XX2, AGAAGGAATCCTGTCAATTCCGAGGAAAGTAATCTGCTTAGGACCT; oligo BN1, AACCATGAATCCCTCCCACAACCC; oligo BN2, AACCATGAATCCCTCCGACTTCCC; oligo β-RARE, CCGGGTAGGGTTCACCGAAAGTTCACTCG; oligo 2*AP-1, GAAACCTGCTGACTCAGATGTCCTGAAACCTGCTGACTCAGATGTCCT; oligo 2*Sp1, AAATAGTCCCGCCCCTAACTCCGCCCAT. For each competition experiment a 100-fold excess of non-radioactive double stranded oligonucleotide was added. For the supershifts with oligo RARE, antibodies against RARα (Ab9α), RARγ1 (Ab1γ1) and RXR (α, β, γ) were kindly provided by C. Rochette-Egly and P. Chambon (Rochette-Egly et al., 1991). After incubating the oligo with nuclear extract for 15 min, 1 μl of ascite fluid antibody was added to the mixture and incubated for another 15 min. After electrophoresis the gels were dried and exposed using a PhosphorImager (Molecular Dynamics).

Acknowledgements

RAR/RXR antibodies were kindly provided by C. Rochette-Egly and P. Chambon and the MEL extracts by Gaetano Zafarana. 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).

References

Andrews, N.C. and Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. 19, 2499.

Boylan, J.F. and Gudas, L.J. (1991) Overexpression of the cellular retinoic acid binding protein I (CRABP-I) results in a reduction in differentiation specific gene expression in F9 teratocarcinoma cells. J. Cell Biol. 112, 965–979.

Bronner-Fraser, M. and Fraser, S.E. (1988) Cell lineage analysis reveals multipotency of some avian neural crest cells. Nature 335, 161–164.

Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. FASEB J. 10, 940–954.

Chen, A.C. and Gudas, L.J. (1996) An analysis of retinoic acid-induced gene expression and metabolism in AB1 embryonic stem cells. J. Biol. Chem. 271, 14971–14980.

de Boer, E., Antoniou, M., Mignotte, V., Wall, L. and Grosveld, F. (1988) The human β-globin gene promoter; nuclear protein factors and erythroid specific induction of transcription. EMBO J. 7, 4203–4212.

de Bruijn, D.R., Oerlemans, F., Hendriks, W., Baats, E., Ploemacher, R., Wieringa, B. and Geurts van Kessel, A. (1994) Normal development, growth and reproduction in cellular retinoic acid binding protein-I (CRABP-I) null mutant mice. Differentiation 58, 141–148.

Dekker, E.J., Vaessen, M.J., van den Berg, C., Timmermans, A., Godsave, S., Holling, T., Nieuwkoop, P., Geurts van Kessel, A. and Durston, A. (1994) Overexpression of a cellular retinoic acid binding protein (xCRABP) causes anteroposterior defects in developing *Xenopus* embryos. Development 120, 973–985.

Dolle, P., Ruberte, E., Kastner, P., Petkovich, M., Stoner, C.M., Gudas, L.J. and Chambon, P. (1989) Differential expression of genes encoding alpha, beta and gamma retinoic acid receptors and CRABP in the developing limbs of the mouse. Nature 342, 702–705.

Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol. 5, 3610–3616.

Gorry, P., Lufkin, T., Dierich, A., Rochette-Egly, C., Decimo, D., Dolle, P., Mark, M., Durand, B. and Chambon, P. (1994) The cellular retinoic acid binding protein I is dispensable. Proc. Natl. Acad. Sci. USA 91, 9032–9036.

Harnish, D.C., Soprano, K.J. and Soprano, D.R. (1992) Mouse conceptuses have a limited capacity to elevate the mRNA level of cellular retinoid binding proteins in response to teratogenic doses of retinoic acid. Teratology 46, 137–146.

Hogan, B., Beddington, R., Constantini, F. and Lacy, E. (1994) Manipulating the Mouse Embryo, A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Horton, C. and Maden, M. (1995) Endogenous distribution of retinoids during normal development and teratogenesis in the mouse embryo. Dev. Dyn. 202, 312–323.

Kothary, R., Clapoff, S., Darling, S., Perry, M.D., Moran, L.A. and Ros-

sant, J. (1989) Inducible expression of an hsp68-lacZ hybrid gene in transgenic mice. Development 105, 707–714.

Lampron, C., Rochette-Egly, C., Gorry, P., Dolle, P., Mark, M., Lufkin, T., LeMeur, M. and Chambon, P. (1995) Mice deficient in cellular retinoic acid binding protein II (CRABPII) or in both CRABPI and CRABPII are essentially normal. Development 121, 539–548.

LeDouarin, N.M. (1982) The Neural Crest, Cambridge University Press, Cambridge, MA.

Leid, M., Kastner, P. and Chambon, P. (1992) Multiplicity generates diversity in the retinoic acid signalling pathways. Trends Biochem. Sci. 17, 427–433.

Leid, M. et al. (1993) Retinoic acid signal transduction pathways. Ann. N. Y. Acad. Sci. 684, 19–34.

Leonard, L., Horton, C., Maden, M. and Pizzey, J.A. (1995) Anteriorization of CRABP-I expression by retinoic acid in the developing mouse central nervous system and its relationship to teratogenesis. Dev. Biol. 168, 514–528.

Lyn, S. and Giguere, V. (1994) Localization of CRABP-I and CRABP-II mRNA in the early mouse embryo by whole-mount in situ hybridization: implications for teratogenesis and neural development. Dev. Dyn. 199, 280–291.

Maden, M., Ong, D.E., Summerbell, D. and Chytil, F. (1988) Spatial distribution of cellular protein binding to retinoic acid in the chick limb bud. Nature 335, 733–735.

Maden, M., Horton, C., Graham, A., Leonard, L., Pizzey, J., Siegenthaler, G., Lumsden, A. and Eriksson, U. (1992) Domains of cellular retinoic acid-binding protein I (CRABP I) expression in the hindbrain and neural crest of the mouse embryo. Mech. Dev. 37, 13–23.

Mader, S., Leroy, P., Chen, J.Y. and Chambon, P. (1993) Multiple parameters control the selectivity of nuclear receptors for their response elements. Selectivity and promiscuity in response element recognition by retinoic acid receptors and retinoid X receptors. J. Biol. Chem. 268, 591–600.

Mangelsdorf, D.J. et al. (1995) The nuclear receptor superfamily: the second decade. Cell 83, 835–839.

Means, A.L. and Gudas, L.J. (1995) The roles of retinoids in vertebrate development. Annu. Rev. Biochem. 64, 201–233.

Nagpal, S., Saunders, M., Kastner, P., Durand, B., Nakshatri, H. and Chambon, P. (1992) Promoter context- and response element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. Cell 70, 1007–1019.

Napoli, J.L., Boerman, M.H., Chai, X., Zhai, Y. and Fiorella, P.D. (1995) Enzymes and binding proteins affecting retinoic acid concentrations. J. Steroid Biochem. Mol. Biol. 53, 497–502.

Norris, A.W., Cheng, L., Giguere, V., Rosenberger, M. and Li, E. (1994) Measurement of subnanomolar retinoic acid binding affinities for cellular retinoic acid binding proteins by fluorometric titration. Biochim. Biophys. Acta 1209, 10–18.

Perez-Castro, A.V., Toth-Rogler, L.E., Wei, L.N. and Nguyen-Huu, M.C. (1989) Spatial and temporal pattern of expression of the cellular retinoic acid-binding protein and the cellular retinol-binding protein during mouse embryogenesis. Proc. Natl. Acad. Sci. USA 86, 8813–8817.

Perez-Castro, A.V., Tran, V.T. and Nguyen-Huu, M.C. (1993) Defective lens fiber differentiation and pancreatic tumorigenesis caused by ectopic expression of the cellular retinoic acid-binding protein I. Development 119, 363–375.

Rochette-Egly, C., Lutz, Y., Saunders, M., Scheuer, I., Gaub, M.P. and Chambon, P. (1991) Retinoic acid receptor gamma: specific immunodetection and phosphorylation. J. Cell Biol. 115, 535–545.

Ruberte, E., Dolle, P., Chambon, P. and Morriss-Kay, G. (1991) Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. Development 111, 45–60.

Ruberte, E., Friederich, V., Morriss-Kay, G. and Chambon, P. (1992) Differential distribution patterns of CRABP I and CRABP II transcripts during mouse embryogenesis. Development 115, 973–987.

Spanopoulou, E., Giguere, V. and Grosveld, F. (1991) The functional domains of the murine Thy-1 gene promoter. Mol. Cell. Biol. 11, 2216–2228.

Umesomo, K., Murakami, K.K., Thompson, C.C. and Evans, R.M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid and vitamin D_3 receptors. Cell 65, 1255–1266.

Vaessen, M.J., Meijers, J.H., Bootsma, D. and Van Kessel, A.G. (1990) The cellular retinoic-acid-binding protein is expressed in tissues associated with retinoic-acid-induced malformations. Development 110, 371–378.

Vasios, G.W., Gold, J.D., Petkovich, M., Chambon, P. and Gudas, L.J. (1989) A retinoic acid-responsive element is present in the 5′ flanking region of the laminin B1 gene. Proc. Natl. Acad. Sci. USA 86, 9099–9103.

Wei, L.N. and Chang, L. (1996) Promoter and upstream regulatory activities of the mouse cellular retinoic acid-binding protein-I gene. J. Biol. Chem. 271, 5073–5078.

Wei, L.N. and Chen, G.J. (1991) Production and analyses of transgenic mice with ectopic expression of cellular retinoic acid-binding protein. Biochem. Biophys. Res. Commun. 179, 210–216.

Wei, L.N., Blaner, W.S., Goodman, D.S. and Nguyen-Huu, M.C. (1989) Regulation of the cellular retinoid-binding proteins and their messenger ribonucleic acids during P19 embryonal carcinoma cell differentiation induced by retinoic acid. Mol. Endocrinol. 3, 454–463.