Overexpression of a cellular retinoic acid binding protein (xCRABP) causes anteroposterior defects in developing Xenopus embryos

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SUMMARY

We have isolated the first Xenopus laevis cDNA coding for a cellular retinoic acid binding protein (xCRABP). xCRABP contains a single open reading frame, coding for an approximately 15×10^3 M_r protein. Northern blot analysis shows that this cDNA hybridizes to a mRNA that is expressed both maternally and zygotically and which already reaches maximal expression during gastrulation (much earlier than previously described CRABP genes from other species). In situ hybridisation showed that at the onset of gastrulation, xCRABP mRNA is localised at the dorsal side of the embryo, in the ectoderm and in invaginating mesoderm. xCRABP expression then rapidly resolves into two domains; a neural domain, which becomes localised in the anterior hindbrain, and a posterior domain in neuroectoderm and mesoderm. These two domains were already evident by the mid-gastrula stage. We investigated the function of xCRABP by injecting fertilized eggs with an excess of sense xCRABP mRNA and examined the effects on development. We observed embryos with clear anteroposterior defects, many of which resembled the effects of treating Xenopus gastrulae with all-trans retinoic acid. Notably, the heart was deleted, anterior brain structures and the tail were reduced, and segmentation of the hindbrain was inhibited. The effects of injecting xCRABP transcripts are compatible with the idea that xCRABP overexpression modulates the action of an endogenous retinoid, thereby regulating the expression of retinoid target genes, such as Hox genes. In support of this, we showed that the expression of two Xenopus Hoxb genes, Hoxb-9 and Hoxb-4, is strongly enhanced by xCRABP over-expression. These results suggest that xCRABP expression may help to specify the anteroposterior axis during the early development of Xenopus laevis.

Key words: cellular retinoic acid binding protein, retinoic acid, protein, anteroposterior defect, Xenopus

INTRODUCTION

There is increasing evidence that vitamin A forms (retinoids) regulate cell differentiation and pattern formation during vertebrate embryogenesis. The best known biologically active retinoid, all-trans retinoic acid (RA), has long been known to be teratogenic in humans (Kochhar, 1967) and has been reported to cause severe birth defects when administered to pregnant women (Lammer et al., 1985). The spectrum of birth defects caused by RA includes cleft palate, specific CNS defects and congenital heart and limb defects, and is conserved in rodents, chickens and frogs suggesting the existence of a well-defined class of RA-sensitive events during vertebrate embryogenesis (Jelinek and Kistler, 1981; Tickle et al., 1982; Thaller and Eichele, 1987; Durston et al., 1989; Sive et al., 1990).

The nature of this RA sensitivity has been investigated in more detail in the developing chick limb (Tickle et al., 1982, 1989; Eichele, 1989), where it is found that a local RA source can mimic a natural organiser region (the zone of polarising activity: ZPA), which specifies the posterior side of the limb bud. The limb bud also contains endogenous RA in an anteroposterior gradient that could define limb polarity in vivo (Thaller and Eichele, 1987).

A second well-investigated event, which is directly relevant for this article, is formation of the main body axis in the early vertebrate embryo. This process is also regulated by an organiser region: the dorsal lip of the blastopore: Spemann’s organiser, in amphibians (Spemann, 1931; Nieuwkoop et al., 1985), or Hensen’s node in birds (Waddington, 1952) and in mammals (Hogan et al., 1992).

A transplanted chicken Hensen’s node can mimic the ZPA in polarising a developing limb bud (Hornbruch and Wolpert, 1986), and Hensen’s node of the chicken embryo has been shown to be an RA source (Hogan et al., 1992; Chen et al., 1992). RA treatment can modify the main A-P axis of developing vertebrate embryos (see, for example Durston et al., 1989; Marshall et al., 1992; Kessel and Gruss, 1991) as well as differentially inducing the expression of class I homeobox-containing genes (Hox genes) (Dekker et al., 1992a,b; Conlon and Rossant, 1992; Marshall et al., 1992). These genes have been implicated in specifying the identities of sequential
antero-posterior zones in the early embryo and in the limb (McGinnis and Krumlauf, 1992). The findings above suggest that active retinoids are important for providing antero-posterior positional information, in the developing limb, and along the main body axis, in early vertebrate embryos.

If active retinoids provide positional information, it is important to understand the mechanisms that regulate their availability and transduce their activity. Until now, much attention has focussed on specific nuclear receptors for retinoids (products of the RAR and RXR gene families). Recent progress in understanding the molecular mechanisms of action of these receptors and their functions in embryogenesis has been discussed recently in some excellent reviews (Leid et al., 1992; Linney, 1992), and will not be explored further here. Another important class of proteins are the cellular retinoid binding proteins which were first identified as cytoplasmic proteins with specific binding affinities for particular retinoids, and the best studied members of which are the cellular retinoic acid binding proteins (CRABPs). Genes encoding two different CRABP isoforms (CRABPI and CRABPII) have now been cloned in mammals and birds, and these prove to code for approximately 15 × 10^3 M_r proteins, with structural similarities to the fatty acid binding protein of the rat and the myelin P2 family of proteins, a family that has been implicated in the transport of specific small hydrophobic molecules. The functions of CRABPs are still unclear: the discussion so far has centered around the possibilities that they have a shuttle function (conveying active retinoids to nuclear receptors; Jetten and Jetten, 1979), that they act as buffers (removing free active retinoids, thus protecting retinoid sensitive structures) or that they participate in retinoid metabolism (Napoli et al., 1991).

Both CRABPI and CRABPII show characteristic temporally restricted expression patterns during chicken and mouse embryogenesis. These patterns, which involve CRABP-specific localisation in axial structures and the developing limb, have been described in detail elsewhere (Maden et al., 1989a,b; Vaessen et al., 1990a,b; Ruberte et al., 1991) and that they are involved in RA metabolism (Napol, et al., 1991).

The purpose of this article is to report the cloning of the first CRABP cDNA in Xenopus laevis and to report on the expression and functioning of the corresponding gene product. Our findings reveal that we have cloned a Xenopus CRABP, which is expressed very early in development. The expression pattern and overexpression phenotype of the corresponding mRNA open the possibility that xCRABP has a function in retinoid-mediated specification of the antero-posterior axis.

### MATERIALS AND METHODS

#### Isolation and sequencing of xCRABP cDNA

A stage 22/24 Xenopus laevis cDNA library, inserted into the EcoRI site of &gt;gt10 (kindly provided by Dr Melton) was plated on Escherichia coli BNN102 and then screened with a mouse CRABPI cDNA probe (Mot-CA11; Vaessen et al., 1990a) and rescreened with two TaqI-TaqI fragments (130 bp and 170 bp) containing the coding region of chicken CRABPI (Vaessen et al., 1990b). The probes were labelled using an Amersham 32P random priming kit (Feinberg and Vogelstein, 1984). The filters were hybridised at 56 °C, in a buffer containing 6× SSC and 9% dextran sulphate, and washed at the same temperature, twice in 3× SSC, 0.1% NaDodSO_4 and twice in 1× SSC, 0.1% NaDodSO_4. Screening approximately 1×10^6 bacteriophages resulted in the isolation of a cDNA clone, designated xCRABP. DNA isolated from xCRABP was digested with EcoRI and ligated into an EcoRI plasmid vector pTZ19R (Pharmacia) and pGEMZf-3 (Promega). Transformation of E. coli DH5α yielded the clone xCRABP, which contains a full-length cDNA insert of 2072 bp. The nucleotide sequence of this insert was determined by the dideoxy termination method (Sanger et al., 1977), using a Promega Sequence kit according to the manufacturer’s instructions.

#### RNA isolation

Xenopus eggs were fertilized in vitro and cultured at room temperature (19-21 °C) in tap water. Synchronous development allowed the collection of many embryos at defined stages (Nieuwkoop and Faber, 1967). The embryos were dejellied using 2% cysteine at pH 7.8. Whole embryos were frozen in liquid nitrogen, then kept in a −80 °C freezer before being homogenised in LiCl-Urea buffer, placed on ice for 18 hours, and pelleted by centrifugation (28,000 revs/minute) for 1 hour. The pellet was then dried and dissolved in TES-buffer (10 mM Tris, 5 mM EDTA and 0.1% NaDodSO_4) and further purified by phenol (pH 7.6) extraction.

#### Northern blotting

For RNA blot analysis, 15 μg RNA samples were electrophoresed on 1% agarose gels in the presence of formaldehyde. After electrophoresis the RNA was transferred to nitrocellulose filters. The filters were hybridised with a 32P labelled probe and were autoradiographed. The RNA blots were hybridised at 65-70 °C in a buffer containing 6× SSC, 9% dextran sulphate, 0.1% herring sperm DNA and washed at 60 °C, twice in 3× SSC, 0.1% NaDodSO_4, and twice in 1× SSC, 0.1 NaDodSO_4.

#### RNase protection assay

DNA templates were made for xCRABP, as well as for the Xenopus laevis S8 ribosomal protein transcript (Xom62/9) (Mariotini et al., 1988). The Xom62/9 template was used to generate an internal standard, to enable quantification of the amount of RNA in each sample. The templates were made using a 138 bp subcloned Sall-EcoRI fragment of xCRABP and an 89 bp fragment of S8 (Xom62/9). For the Xenopus Hoxb genes, a 153 bp template of Hoxb-4 (Xhoxb-4) and a 227 bp template of Hoxb-9 (Xhboxb9) were used (Dekker et al., 1992a). These were inserted into CspCl gradient-purified pGEMZf-3 (Promega) recombinants. Sp6 RNA polymerase was then used to transcribe the templates.
make antisense RNA probes (labelled via α-32P UTP), as required for the RNase protection assays. The probes were first treated with DNase I and then purified on a 7% urea-acrylamide gel. In the RNase protection assays, mixtures of the two labelled antisense probes (of xCRABP plus the internal standard) were used (at 45°C) to hybridise to RNA extracted from embryos or fragments, protecting their homologous transcripts from digestion by a mixture of 2 µg/ml RNase T1 (Pharmacia) and 40 µg/ml RNase A (Life Technology). All the procedures were basically as described by Melton et al. (1984), with slight modifications as described by Simeone et al. (1990). The gels were then analysed directly using a phosphorimager (Molecular Dynamics, Compaq, Image Quant (Desk 386/25e) to measure the radioactivity corresponding to the protected fragment. The radioactive label corresponding to the xCRABP gene and to the internal standard (Xom62/9) was thus quantified and the value for xCRABP for each sample was then normalized to the value for the corresponding internal standard. The same was perfomed for Hoxb-4 and Hoxb-9 together with Xom62/9 as internal standard in each lane (Fig. 8). These normalized values were used to generate the data shown (Fig. 4A).

In situ hybridisation

The procedure for whole-mount in situ hybridisation was essentially as described by Harland (1991), with minor modifications (A. Koster, unpublished data). A linearised template of xCRABP (an EcoRI-SalI fragment and a SalI-EcoRI fragment (respectively of 1050 bp and 950 bp, both linearised with HindIII) was used for in vitro transcription reactions together with digoxigenin-11-UTP (Boehringer Mannheim 1209 256) and SP6 RNA polymerase (as described by Melton et al., 1984). The whole-mount embryo preparations were examined using a Zeiss Axiosvert 35 microscope. The embryos were later embedded in paraffin and then sectioned. The sections were examined using a Zeiss Axiovert 35 microscope. The embryos were then examined under an Olympus BH-2 microscope using dark-field illumination and photographed.

Synthesis of mRNAs

xCRABP full-length mRNAs were synthesized using Sp6 or T7 RNA polymerase together with digoxigenin-11-UTP (Boehringer Mannheim) and biotinylated (as described by Melton et al., 1984). The whole-mount embryo preparations were incubated in 1% trichloracetic acid (TCA) for 2 hours and then washed three times for 15 minutes in PBS buffer. Embryos were incubated overnight in 1% bovine serum albumin (BSA), 0.2% polyoxyethylene sorbitan monolaureate (Tween 20) in PBS. A 1:5 dilution of the 2G9 antibody in PBS-Tween 20 was added and incubated overnight at 4°C. The next day the embryos were washed in PBS-Tween 20 (whole day). A 1:200 diluted biotinylated anti-mouse IgG was added (incubation overnight) and embryos were washed in PBS-Tween 20 for 8 hours. Thereupon, a 1:200 streptavidin-FITC coupled antibody was added for some hours or overnight. Embryos were then washed in PBS-Tween 20, washed twice in methanol and twice in Murrays.

The embryos were then examined using a confocal scanning laser microscope and photographed.

Graphical reconstruction

Stage 47 xCRABP-injected and control embryos were processed for histology, sectioned transversely and stained with hematoxylin-eosin (see above). The graphical reconstruction of the embryo on a sagittal plane was done using two co-ordinates. Vizualised in transverse sections, a frontal plane (ventral surface of the embryo), functions as the base line. The sections are orientated so that the vertical median plane of the sections is perpendicular to the base line. Both lines are parallel to the A-P axis of the embryo. These planes give two perpendicular lines in every cross-section, a horizontal and a vertical line that can be used as a base for reconstruction. The outlines of the body and organs are then plotted using the position of the CNS as a reference point throughout the entire length of the embryo. This method of reconstruction (projection on a sagittal plane) can only be used if the median line of the brain, as well as the middle of the roof of the mouth cavity can be determined in every head section. In the remainder of the embryo, the dorsal and the ventral boundaries of the spinal cord were used as reference points.

RESULTS

Cloning and expression of a Xenopus CRABP (xCRABP)

We obtained a full-length Xenopus laevis CRABP cDNA clone by screening an embryonic stage 22/24 cDNA library with a full-length 800 bp EcoRI-EcoRI mouse CRABP clone (Vaessen et al., 1990a), a 170 bp TaqI-TaqI fragment and a 130 bp EcoRI-TaqI fragment from mouse CRABP and a 314 bp partial chicken CRABP clone (Vaessen et al., 1990b), all containing part of the open reading frame (ORF). This screen yielded a 2027 bp bacteriophage cDNA clone: xCRABP, which cross hybridised to mouse and chicken CRABP on Southern blots, and also resembled mouse and chicken CRABP cDNA probes in hybridising to an approximately 1 kb transcript on a northern blot of total RNA from murine P19EC-derived ME-1 cells (not shown). This cDNA was cloned into the EcoRI site of pGEM3Zf (+) and, when sequenced, proved to contain an ORF from nucleotide 130 to nucleotide 495. The ORF encodes 155 amino acids: nine amino acids longer than the other known CRABPs (Fig. 1A). xCRABP is 82.2% homologous, at the amino acid level, with mouse CRABP (Vaessen et al., 1990a),
83% with bovine CRABPI (Shubeita et al., 1987), 79.5% with chicken CRABPI (Momoi et al., 1990) and 82.5% with mouse CRABPI. Ignoring conservative amino acid substitutions, the homology with both mouse CRABPs is 92%. This ORF thus encodes a typical CRABP which, based on sequence comparison, cannot be classified either as CRABPI or as CRABPII. Sequencing the complete EcoRI fragment revealed further that the mRNA for xCRABP differs from other known CRABP transcripts in containing extensive 3’ and 5’ untranslated sequences. Northern blots of total Xenopus RNA (see below) also confirmed that the xCRABP transcript is much larger than the 1 kb transcripts so far reported for other CRABPs. The facts above and the unusual expression pattern of xCRABP (below), may well mean that we have cloned a novel CRABP, but we prefer to be cautious in making this interpretation.

The expression of xCRABP was followed by hybridising a specific 3’ untranslatable trailer sequence to northern blots containing 15 µg of total RNA samples isolated from a series of Xenopus developmental stages. The blot was rehybridised to a random-primed histone H3 probe (Destrée et al., 1984), as a control for RNA loading (Fig. 2). This analysis revealed a 2.3 kb transcript, which was available at a low level in oocytes, decreasing rapidly to a very low but still detectable level in early developmental stages (Fig. 2). At the start of gastrulation (stage 10), the availability of this xCRABP transcript began to increase rapidly, reaching maximum expression in the mid- to late gastrula (stages 11-12), before decreasing gradually during later development, so that the transcript was still present at a high level at the latest stage examined (tadpole; stage 35). These results with northern blotting were also confirmed using RNase protection assays (Fig. 4A).

We note that the xCRABP transcript is expressed much earlier in development than other (mouse and chicken) CRABP transcripts, whose expression has been described previously (Ruberte et al., 1992; Vaessen et al., 1990a,b). The expression period for xCRABP coincides in time with a part of the retinoic acid-sensitive period for anteroposterior specification in early Xenopus development (Durston et al., 1989; Sive et al., 1990).

**In situ hybridisation: localised expression of xCRABP in the dorsal marginal zone of gastrula stage embryos**

The spatiotemporal expression pattern of xCRABP was examined by using whole-mount in situ hybridisation with digoxigenin-labelled RNA probes, visualised using an alkaline phosphatase-conjugated anti-digoxigenin antibody with nitro blue tetrazolium (NBT) as the substrate (Harland, 1991). The specificity of the in situ hybridisation signal was tested by using two different antisense xCRABP probes (a 3’ untranslated 1050 bp EcoRI-SalI fragment as well as a 5’ translated 950 bp SalI-EcoRI fragment) as well as the sense versions of these probes (as negative controls). The expression patterns obtained using the 3’ untranslated probe and the 5’ translated probe (see Fig. 1B) were identical. Sense xCRABP mRNA digoxigenin-labeled probes never showed a hybridisation

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**Fig. 1.** The corresponding amino acid sequences for xCRABP and the xCRABP cDNA probes and artificial xCRABP mRNAs used in this study. (A) Alignment of one letter code protein sequence of the Xenopus laevis cellular retinoic acid binding protein (xCRABP) with those of known CRABPs from other species. Xenopus laevis CRABP is compared with bovine CRABPI (bCRABPI) (Shubeita et al., 1987), and with mouse CRABPI (mCRABPI) (Vaessen et al., 1990a), mouse CRABPII (mCRABPII) (Kitamoto et al., 1989) and a partial chicken CRABPIII amino acid sequence (cCRABPII-I) (Vaessen et al., 1990b). Boxed sequences indicate non-conservative amino acid substitutions. (B) Restriction map of the xCRABP cDNA clone. The black box indicates the open reading frame which is the region homologous to other known CRABPs (shown in Fig. 1A). The lower panel shows the probes used in our experiments. For northern blot analysis and in situ hybridisation a 1050 bp EcoRI/SalI open reading frame containing fragment (1) and a 950 bp SalI/EcoRI non-coding fragment (2) of xCRABP cDNA were used as probes. A 138 bp template (SalI-AvalII fragment) was used to generate antisense RNA xCRABP probe for RNase protection assays (3). (C) Diagrammatic representation of the sense (1), antisense (2), and nonsense (3) 3’ end of the mRNA, generated by polymerase-generated mRNAs used for injection into 1-cell-stage Xenopus embryos and used in the rabbit reticuloocyte lysate assay. The nonsense mRNA contains an in-frame 270 bp human parathyroid hormone receptor (PTHR) Aval fragment (Karperien et al., unpublished data) cloned in the Aval site of xCRABP.
signal (not shown). xCRABP hybridisation was non-detectable in situ in very early developmental stages (stage four to early blastula). Presumably, the mRNA concentrations in these early stages are below the detection limit for this in situ hybridisation method. By gastrulation (stage 10.5; Fig. 3A), xCRABP transcripts were found in the cytoplasm and were concentrated in the dorsal marginal zone (Spemann organiser), and the dorsal part of the animal cap. By the mid-gastrula stage (stage 11.5; Fig. 3B), xCRABP expression was concentrated into two main domains; a posterior domain in the dorsal lip of the blastopore and a more anterior expression domain in the mid neural plate. These anterior and posterior expression domains were conserved in later (neurula and tailbud) stages, the anterior domain becoming localised in the posterior hindbrain and the posterior domain in the tail (both in mesodermal and neural structures) (stage 15, Fig. 3C). At tailbud stages (stages 25 and 32; Figs 3D, 3E and 3F), the hybridisation signal was found in the branchial arches, the tip of the tail, the hindbrain, in rhombomere 4, above the otic vesicle and in rhombomeres 6, 7 and 8, and in the anterior spinal cord. The anterior spinal cord, next to the hindbrain, showed a relatively high hybridisation signal which quickly faded out posteriorly. Furthermore, positive staining could be observed in the region of the nasal groove and at the boundary between the diencephalon and the mesencephalon. The expression pattern of xCRABP is thus complex. The striking early anterior and posterior localisation zones of xCRABP in the gastrula and neurula are consistent with the idea that this gene could have a function in transducing retinoid-mediated specification of the anteroposterior axis, since the developing anteroposterior axis is sensitive to RA treatment during these stages.

RA and xCRABP expression

It is known that RA treatment enhances CRABPII expression in mouse cell lines (Durand et al., 1992) and that it can repress expression of CRABPI in mouse cell lines (Vaessen et al., 1990b). We therefore examined the effect of continuous treatment (from the 1-cell stage onwards) with 10⁻⁶ M RA on the expression of xCRABP mRNA in Xenopus embryos, by using in situ hybridisation. This RA concentration was previously found to be effective in modulating the expression of RA-responsive genes in early Xenopus embryos (Ruiz i Altaba and Jessell, 1991; López and Carrasco, 1992; Dekker et al., 1992a), and in generating axially deficient embryos (Durston et al., 1989; Sive et al., 1990).

The in situ data (Fig. 3G,H) show that RA treatment of early gastrula stage embryos alters the xCRABP expression pattern. xCRABP expression is clearly enhanced in the anterior part of the mid-neurula-stage embryo (stage 14-15), but the expression is repressed posteriorly (Fig. 3G). Later, at the tailbud stage (stage 25), enhanced xCRABP expression is generally observed both in the anterior part of the embryo and in the tail (Fig. 3H). This contrasts with the situation in control (non-treated) embryos, where xCRABP expression is now restricted to specific regions in the posterior part of the hindbrain, branchial arches, etc. (see above; Fig. 3E,F), and where a lower expression level is observed in the tip of the tail. RA treatment thus seems to enhance xCRABP expression generally, but to reduce posterior expression of xCRABP in the tail. These findings show that RA can modulate the axial pattern of xCRABP expression.

Overexpression of xCRABP causes anteroposterior defects

Because the timing and localisation of xCRABP expression allow the possibility that xCRABP is involved in retinoid-mediated anteroposterior specification, we were interested in testing this idea by manipulating the availability of xCRABP mRNA. We therefore set up overexpression experiments, by injecting synthetic sense xCRABP mRNA into the Xenopus zygote, and by injecting synthetic antisense and nonsense xCRABP mRNAs as negative controls. The synthetic xCRABP mRNAs used in this study are shown in Fig. 1C. The sense xCRABP mRNA contains the entire coding region for the xCRABP protein. The antisense mRNA contains the same insert but is transcribed in the antisense orientation. The nonsense xCRABP mRNA contains an AavI PTHR fragment (see experimental procedures and Fig. 1C), which was cloned in-frame into the ORF of xCRABP. These three mRNAs were translated in vitro using a rabbit reticulocyte lysate and each proved to give an appropriately sized product (not shown). Anti-sense xCRABP mRNA provides a suitable negative control in this study because of the known instability of antisense mRNAs after injection into the Xenopus zygote (reviewed by Woolf, 1992).

The stability of the injected mRNA during early development is of key importance in these studies. If the sensitive period (with respect to xCRABP overexpression) is the period in which endogenous xCRABP mRNA expression is high (i.e. during gastrulation and thereafter), then injected RNA must be stable enough to last through gastrulation. We injected about 2-4 ng synthetic sense xCRABP mRNA into 1-cell-stage embryos, an amount below that which induces toxic effects during cleavage (Rebagliati and Melton, 1987). The stability of the sense xCRABP mRNA was then measured by perform-
ing RNase protection assays, using total RNA from various stages after microinjection (Fig. 4A). These data were also confirmed and extended by northern blots of total RNA from late neurula embryos (stage 20) (Fig. 4B), which showed abundant availability of a 2.0 kb \textit{xCRABP} mRNA, corresponding to the size of the synthetic mRNA which was injected. The results indicate that \textit{xCRABP} mRNA has a half life of approximately 20 hours in developing embryos and thus appears highly stable. Significantly, the amount of injected mRNA remaining in late neurula stage embryos (stage 20) (approximately 10% of what was injected) represents a 100-fold excess over the endogenous level of \textit{xCRABP} mRNA present at that stage and confirms that injected \textit{xCRABP} mRNA persists through gastrulation and neurulation, well beyond the time when the endogenous \textit{xCRABP} gene is first expressed (mid-gastrulation) (Fig. 4A-B).

Fig. 5 shows that injection of 2 ng of sense \textit{xCRABP} mRNA induces developmental defects. Over 63% of the injected zygotes (515 embryos) developed to larvae with severe defects. Very few (5-7%) of the control larvae (472 embryos; from zygotes injected with TE-buffer (10 mM Tris and 5 mM EDTA), antisense (see Woolf, 1992, for review) or nonsense \textit{xCRABP} mRNA) developed any defective structures. All defective \textit{xCRABP}-injected larvae showed clear tail defects (kinked or shortened tails). Many also showed clear anterior abnormalities with reduced or fused eyes, and strongly reduced cement glands (Fig. 6A-D). We therefore decided to study the effect of \textit{xCRABP} mRNA injection on the development of neural tissues by immunostaining stage 47 embryos (tadpole) with a neural-specific antibody, 2G9, and comparing them with un.injected embryos (Fig. 6E-F). The results revealed that, while the hindbrain often appears relatively normal, forebrain structures are usually strongly reduced. Furthermore, it was also clear that the further outgrowth of the spinal cord into posterior domains of the tail was inhibited. Injection of lower doses of \textit{xCRABP} mRNA (1-0.5 ng) induced progressively less severe but qualitatively similar defects in head and tail development.

We made a detailed histological analysis of \textit{xCRABP} mRNA-injected embryos (2 ng) to characterise further the \textit{xCRABP} phenotype. Histological sections were prepared from injected and control embryos reared to a tadpole stage (stage 47). At this developmental stage, organs and tissues in control embryos are well defined and easily recognized. The \textit{xCRABP}-injected embryos also sometimes survived to this stage and their malformations could then be characterized in detail. We used a specific method of reconstruction: projection onto a sagittal plane (Nieuwkoop, 1947). This enabled us to make detailed graphical reconstructions of the injected embryos (Fig. 7).

Generally, most of the normal embryonic structures were present in \textit{xCRABP}-injected embryos although some were reduced and/or malformed. One exception was the embryonic heart, which was lacking or strongly reduced in all \textit{xCRABP} mRNA-injected embryos examined. The embryos also lack blood circulation, but the pericardium is always present. It is obvious that \textit{Xenopus} embryos do not require a functional heart and blood circulation for early development, at least up to stage 47. As described above, anterior neural structures were also reduced. The telencephalon, diencephalon and mesencephalon were recognizable, but strongly reduced. The rhombencephalon was not dramatically reduced in size but segmentation into rhombomeres was apparently absent. As described above, the eyes were also abnormal, often being fused and usually lacking an optic nerve and lens. The most extreme \textit{xCRABP} phenotype had only one primary eye cup at the anterior end of the neural tube with no optic nerve, lens or other eye associated structure. Another diencephalon-related structure, the pineal body, was generally absent. Surprisingly, one anterior structure, the nasal pit, was exceptional in being duplicated. Two pairs of nasal pits were sometimes present. The notochord often bifurcated and extended too far anteriorly, reaching as far as the eye cup. The gut failed to coil properly, so that it extended further posteriorly than usual and was sometimes enlarged. It also usually lacked a mouth or an anus.

![Fig. 3. \textit{xCRABP} in situ hybridisation. Whole embryos were hybridised to antisense \textit{xCRABP} probes. Hybridisation was visualised using an alkaline phosphatase reaction with nitro blue tetrazolium (NBT) as the substrate. (A) Stage 10.5 (early gastrula stage), cross section. Staining was observed in the animal hemisphere and in the marginal zone above the dorsal lip (DL). (B) Stage 11.5 (late gastrula embryo), dorsal view. Staining is evident in the presumptive anterior (hindbrain region) part of the embryo. A semicircle of staining was also found in the dorsal lip (DL) of the blastopore, in the posterior region of the developing embryo. It is notable that, already at this stage, the midline of the neural plate (notoplate) shows lower \textit{xCRABP} expression. (C) Stage 14/15 (early neurula stage), dorsal-lateral view. Staining was found in the presumptive hindbrain and tail regions. There are also small lateral and anterior patches of staining, which possibly correspond with the future nasal pit and branchial arch regions. The midline of the neural plate is negative for \textit{xCRABP} expression. (D) Stage 25 (tailbud stage), dorsal view. This photo shows regional differences in the distribution of \textit{xCRABP} mRNA in the CNS. Anteriorly, the forebrain-related structures show very light staining, the midbrain structures show somewhat stronger staining while the hindbrain shows abundant \textit{xCRABP} expression. The spinal cord also clearly shows positive staining. (E) Stage 25 (tailbud stage), side view (same embryo as shown in Fig. 3D). \textit{xCRABP} expression is evident in the tail region (in the mesoderm as well as in the central nervous system), in the neural tube, in the hindbrain region, and in the presumptive branchial arch and nasal pit area. (F) Stage 32 (tailbud stage), side view. The hybridisation signal was found in the branchial arches (BA), as well as the hindbrain, in rhombomere 4 above the otic vesicle (OV), in rhombomeres 6, 7, and 8 (rhombomere 5 shows no \textit{xCRABP} expression) and in the anterior spinal cord. The anterior spinal cord, next to the hindbrain, demonstrates a relatively abundant signal that fades in the more posterior part of the spinal cord. Furthermore, staining can be observed in the region of the nasal pit (NP) and at the boundary between the mesencephalon and the rhombencephalon. The tip of the tail also still contains \textit{xCRABP} mRNA. (G) An RA-treated stage 14/15 (early neurula stage) embryo, side view. Enhanced expression of \textit{xCRABP} was observed in the presumptive hindbrain region of the embryo. No staining was observed in the presumptive tail region. This embryo, and the embryo in Fig. 3H, were stained for a shorter time than the embryos in Fig. 3A-G (see below). The anterior hybridisation signal is actually much stronger than in control embryos at the same stage. (H) A RA-treated stage 25 (tailbud stage) embryo, side view. Enhanced expression of \textit{xCRABP} occurs both in the presumptive hindbrain region and in the tail (staining as in Fig. 3G). In B-H, the embryos are shown with their anterior sides to the left. Note: The embryo depicted in A was stained for 3 days, while the embryos shown in B-F were stained overnight with NBT (see experimental procedures).]
and the tail was also markedly malformed. The notochord ends posteriorly in a ‘knot-like’ structure. Possibly it is inhibited from extending, but still proliferates, leading to the formation of this structure. The neural tube, which normally follows the outgrowth of the notochord into the tail, is also truncated and bifurcates at the level of the ‘knot-like’ notochordal structure. In addition, the kidneys were often underdeveloped.

Taken together, these observations reveal that xCRABP-injected embryos show many defects, most of which concern development of anterior and posterior structures. The defects show quite a strong resemblance to those that have previously been described in embryos that were treated with RA during gastrulation (Durston et al., 1989; Sive et al., 1990; Papalopulu et al., 1991). Notably, both RA treatment and xCRABP overexpression caused reduction of anterior neural structures, deletion of the heart, and inhibition of rhombomere segmentation and tail development. It appears that overexpression of xCRABP is non permissive for normal anteroposterior development. Possibly it interferes with the functioning of an endogenous retinoid signal.

**xCRABP overexpression enhances the expression of Hoxb genes**

The target genes for retinoids in the early embryo are not known, but there is much evidence that class I homeobox-containing genes (Hox genes) are involved in anteroposterior patterning during early vertebrate embryogenesis (reviewed in McGinnis and Krumlauf, 1992). These genes are also differentially induced by RA (Simeone et al., 1990), and ectopic Hox gene expression patterns induced in early vertebrate embryos by exogenous RA treatment correlate with, and possibly cause, specific anteroposterior transformations (Kessel and Gruss, 1991; Marshall et al., 1992; Holder and Maden, 1992).

These considerations make it interesting to examine the effects of xCRABP overexpression on Hox gene expression during early Xenopus development. We therefore performed RNase protection assays to measure the expression of two Xenopus Hoxb genes (Hox-2 genes) in xCRABP-injected embryos and in control (uninjected and anti-sense mRNA-injected) embryos (Fig. 8). The experiment shows that expression of both Hox genes is hyperinduced by xCRABP injection. Hoxb-4 (Xhox-1A) is more strongly hyperinduced compared to Hoxb-9 (XlHbox6) at both of the stages investigated. This effect of xCRABP overexpression resembles the effect of treating gastrula stage embryos with a high concentration (10^-6 M) of RA. These observations fit with the idea

![Fig. 4](image_url). The stability and the translation of the xCRABP mRNA. (A) RNase protection assays were used to examine the stability of injected sense xCRABP mRNA during development. These were used to measure xCRABP mRNA-injected embryos (hatched bars) and controls injected with full-length antisense xCRABP mRNA (open bars) at the stages (Nieuwkoop and Faber, 1967) indicated using a 138 bp xCRABP 3′ untranslated (SalI-AvalI) fragment as the probe (see Fig. 1B, fragment 3). Xom62/9 was used as an internal standard for quantification. The sense xCRABP-injected embryos maintain a strongly enhanced level of xCRABP mRNA. (B) A Northern blot showing three lanes with total RNA, isolated at stage 20 (late neurula/early tailbud stage) from non-injected (1), sense (2) and antisense xCRABP (3)-injected embryos hybridised to a 950 bp 3′ untranslated SalI-EcoRI fragment of the xCRABP cDNA clone. This also demonstrates the stability of the 2.0 kb synthetic mRNAs injected into 1-cell-stage embryos. (C) Translation of xCRABP mRNA. A rabbit reticulocyte assay (Promega) and metabolic labeling using [35S]methionine was used to confirm that the sense xCRABP mRNA is translated into an approximately 15x10^3 Mr protein (lane 1). The antisense xCRABP mRNA is not translated into a protein (lane 2).

![Fig. 5](image_url). sense xCRABP mRNA overexpression causes developmental defects. Microinjection of sense xCRABP mRNA (A), TE-buffer (B), antisense xCRABP mRNA (C) and nonsense xCRABP mRNA (D) into 1-cell-stage Xenopus embryos. The injection of sense xCRABP mRNA (A) induced anteroposterior defects in a large percentage (63%) of the injected embryos, while the controls (B-D) failed to develop many defects. The embryos were examined at stage 35 for specific A-P defects, e.g. reduced or fused eyes, reduced cement gland, reduced forebrain structures, tail shortening, reduction of heart and kidneys (see experimental procedures). Values are means ± s.e.m. of 9 independent experiments.
that xCRABP could be important for transducing an early retinoid signal. A further surprising observation was that treatment of xCRABP-injected embryos with RA caused no further increase in Hox gene expression. Possibly xCRABP injection alone is sufficient to raise Hox gene expression to a maximum level.

Fig. 6. Phenotypes of normal and xCRABP-injected embryos. A, anterior; P, posterior. (A) Normal stage 35 (tailbud stage) embryo, side view. (B) xCRABP mRNA-injected stage 35 embryo, side view. (C) Normal stage 47 (tadpole) embryo, side view. (D) xCRABP mRNA-injected stage 47 embryo, side view. (E) Stage 47 embryo, dorsal view. Stained with 2G9, a neural-specific antibody to reveal the central nervous system. FB, forebrain; HB, hindbrain; SC, spinal cord. (F) xCRABP mRNA-injected stage 47 embryo, dorsal view, stained with 2G9. This staining shows that the forebrain (FB) is strongly reduced, the hindbrain (HB) is still detectable, but the spinal cord (SC) ends up in a knot-like structure and does not develop in the posterior (tail) region of the embryo.
DISCUSSION

The first *Xenopus* cellular retinoic acid binding protein: (xCRABP)

We report the cloning of a cDNA for the first identified *Xenopus* CRABP gene: xCRABP. The open reading frame of this cDNA displays a high level of homology (92%), at the protein level with each of the two known murine CRABPs (CRABP I and CRABP II), as well as with the known avian and bovine CRABPs (Fig. 1A), if conservative amino acid substitutions are ignored. The coding sequence of this CRABP thus failed to allow its identification as either of the known CRABP types and suggested that we could be dealing with a novel CRABP. However, since this is the first amphibian CRABP cDNA isolated, the sequence data do not rule out that xCRABP is a functional homologue of a previously described CRABP type. Information about the function of xCRABP was provided by analysis of its spatiotemporal expression pattern, and by functional analysis via overexpression of its mRNA (below).

xCRAB has a unique, early, axially localised expression pattern

xCRAB showed a complex spatiotemporal expression pattern that differs from the published expression patterns of CRABP I in the mouse and of CRABP II in the mouse and the chicken. For example, xCRAB resembles mouse CRABP I and CRABP II in showing localised expression in the central nervous system, including an expression zone in the hindbrain, but differs from both of these genes in the details of this pattern. At the tailbud stage xCRAB expression in the hindbrain was concentrated in rhombomeres 4 (above the otic vesicle) and 6, 7 and 8, but was undetectable in more anterior rhombomeres. At a comparable developmental stage in the mouse (11-12 somite stage), CRABP I is expressed in rhombomeres 4, 5 and 6, and at low levels in the other rhombomeres. At the same stage mouse CRABP II is expressed at a high level in rhombomere 4, but not in rhombomere 5, and only at a low level in the other rhombomeres (Ruberte et al., 1992). There are other similarities to details of the expression patterns of each of the known CRABPs, but also other differences from both. For example, xCRAB resembles mouse CRABP II in being expressed in the heart, in the telencephalic, diencephalic, mesencephalic and metencephalic regions, in the cranial ganglia, in the eye region and in the spinal cord. It resembles mouse CRABP I in being expressed in nasal groove, neural crest, branchial arches, heart, digestive and pharyngeal mesenchyme. It differs from both in other details, especially its expression at very early stages. Considering the strongly conserved organisation of structures such as the hindbrain, this unique expression pattern of xCRAB may mean that it has a specific novel function, but we prefer to be cautious in claiming that this is a novel CRABP. It would be interesting to see whether homologues of xCRAB can be identified in other vertebrates. In this respect, it is of interest to point out that hybridisation of the xCRAB cDNA to a northern blot containing mRNA from different developmental stages of the zebrafish revealed a transcript that is similar in size to, and is expressed with developmental timing similar to, xCRAB mRNA (not shown).

A unique feature of xCRAB expression is that it starts very early in development: xCRAB mRNA is available in the zygote, is subsequently degraded, but is transcribed again from the beginning of gastrulation and reaches maximal expression by the mid-gastrula stage. Expression of xCRAB is thus initiated much earlier than that of murine CRABP I or CRABP II (mRNA detectable in the mouse from 5-7 somites, neurula stages; Ruberte et al., 1992). Furthermore, this early high expression level of xCRAB is of particular interest because it overlaps in time, with part of an early RA-sensitive period, during which RA can modify the main anteroposterior axis of the developing *Xenopus* embryo. This correlation raises the possibility that xCRAB has a role in transducing or modulating a part of this RA effect; a possibility that is reinforced by the early axially localised pattern of xCRAB mRNA expression. Namely, transcripts were concentrated at the dorsal side in the animal cap and in the dorsal marginal zone of the early gastrula, becoming posteriorly localised in the late gastrula stage with a secondary more anterior expression zone developing in the mid-neural plate in the presumptive hindbrain region, by the mid-gastrula. This expression pattern
makes it interesting to consider the idea that xCRABP expression is involved in transducing and/or modulating the effects of an endogenous retinoid signal that specifies the early anteroposterior axis and it is worth noticing that the development of an xCRABP expression zone in the mid-neural plate is one of the earliest markers so far described for regionalisation of the central nervous system. The timing of the genesis of this anterior zone correlates well with the time when regional neural differences are first specified in the amphibian embryo (Nieuwkoop et al., 1952). The results of RA treatment suggest further that localised endogenous retinoids may modulate the expression of xCRABP itself. Our findings here also add one more putative regulatory gene to the list, including those for the transcription factors goosecoid (Cho et al., 1991), XLIM-1 (Taira et al., 1992) and XFKH1 (Dirksen and Jamrich, 1992), and for secreted proteins like noggin (Smith and Harland, 1992), which show localised expression in the Spemann organiser.

**xCRABP overexpression causes specific axial defects**

After microinjecting excess xCRABP mRNA, we observed a spectrum of axial defects, which were not obtained in negative controls (i.e. in embryos injected with antisense or nonsense xCRABP mRNAs or with TE-buffer). Most notably, development of anterior structures (including forebrain structures, mouth and heart) and of posterior structures (tail, posterior notochord and neural tube, the anus and the kidney) was inhibited and/or abnormal. These results indicate that xCRABP overexpression is non-permissive for normal anteroposterior patterning, and they reinforce our conclusion from the expression pattern of xCRABP that it will be interesting to investigate whether this gene has a role in anteroposterior patterning. Our results showed further that the expression of two mid-body-specific Hox genes was also enhanced. Considering the mounting evidence that combinatorial expression patterns of Hox genes specify anteroposterior identity, it is likely that the anteroposterior transformations observed following xCRABP mRNA injection are mediated, at least partly, via effects on Hox gene expression. Many authors have also suggested that CRABPs have a specific role in regulating the expression of Hox genes during development (Ruberte et al., 1992; Denker et al., 1990; Vaessen et al., 1990a,b; Dollé et al., 1992; Taira et al., 1992; Dirksen and Jamrich, 1992), and for secreted proteins like noggin (Smith and Harland, 1992), which show localised expression in the Spemann organiser.

**Fig. 8.** xCRABP mRNA overexpression enhances Hoxb gene expression during early development. (A) 1-cell-stage embryos were injected with antisense xCRABP mRNAs (B), sense xCRABP mRNAs (C), and treated with $10^{-6}$ M RA continuously from stage 10 onwards (D) and isolated at stage 15 (mid neurula) and 20 (late neurula) together with non-injected embryos at the same stages (A) and from the same batch of embryos. Total RNA was extracted from these embryos and used to perform an RNase protection assay using two Xenopus laevis Hoxb (Hox-2) genes and an internal standard Xom62/9 gene (see experimental procedures). Hoxb-9 (XHbox6) is the most 5' localised Hoxb gene and Hoxb-4 (Xho-1A) is a 3' localised gene in the Hoxb chromosomal complex (Dekker et al., 1992a). Note: We have adopted the Xenopus Hox gene nomenclature according to a recent proposal by M. P. Scott (1993) Cell 71, 551-553. (B,C) Quantification of Hoxb gene expression. The results shown in A were quantitated by phosphorimaging. Open bars, control embryos; lightly hatched bars, anti-sense xCRABP-injected embryos; heavily hatched bars, sense xCRABP-injected embryos; black bars, RA-treated embryos. Expression of Hoxb-4 (B) and Hoxb-9 (C) was measured by RNase protection at the developmental stages indicated. Xom62/9 was used as internal standard. Data are expressed as percentages relative to the maximum expression of Hoxb-4 and Hoxb-9 during development. The results show that expression of both Hox genes is hyperinduced by xCRABP injection: Hoxb-4 (Xho-1A) is 3-fold hyperinduced at stage 15, and 2-fold hyperinduced at stage 20, and Hoxb-9 (XHbox6) is 2-fold hyperinduced at stage 15 and 1.15-fold hyperinduced at stage 20. This effect of xCRABP overexpression resembles the effect of treating gastrula stage embryos with a high concentration ($10^{-6}$ M) of RA.
REFERENCES


CRABP expression and anteroposterior defects in Xenopus