Retinoic acid-binding protein, rhombomeres and the neural crest

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Summary

We have investigated by immunocytochemistry the spatial and temporal distribution of cellular retinoic acid-binding protein (CRABP) in the developing nervous system of the chick embryo in order to answer two specific questions: do neural crest cells contain CRABP and where and when do CRABP-positive neuroblasts first arise in the neural tube? With regard to the neural crest, we have compared CRABP staining with HNK-1 staining (a marker of migrating neural crest) and found that they do indeed co-localise, but cephalic and trunk crest behave slightly differently. In the cephalic region in tissues such as the frontonasal mass and branchial arches, HNK-1 immunoreactivity is intense at early stages, but it disappears as CRABP immunoreactivity appears. Thus the two staining patterns do not overlap, but are complementary. In the trunk, HNK-1 and CRABP stain the same cell populations at the same time, such as those migrating through the anterior halves of the somites. In the neural tube, CRABP-positive neuroblasts first appear in the rhombencephalon just after the neural folds close and then a particular pattern of immunoreactivity appears within the rhombomeres of the hindbrain. Labelled cells are present in the future spinal cord, the posterior rhombencephalon up to rhombomere 6 and in rhombomere 4 thus producing a single stripe pattern. This pattern is dynamic and gradually changes as anterior rhombomeres begin to label. The similarity of this initial pattern to the arrangement of certain homeobox genes in the mouse stimulated us to examine the expression of the chicken Hox-2.9 gene. We show that at stage 15 the pattern of expression of this gene is closely related to that of CRABP. The relationship between retinoic acid, CRABP and homeobox genes is discussed.

Key words: cellular retinoic acid-binding protein, retinoic acid, rhombomeres, neural tube, neural crest, Hox-2.9, homeobox.

Introduction

The hypothesis that retinoic acid (RA) is a morphogen in vertebrate development is an amalgamation of two lines of evidence: the effects of administration of excess RA to the developing embryo and the identification of RA as an endogenous component of the embryo. The two developing systems that have so far been investigated in this regard are the limb and the nervous system.

In the developing chick limb bud, when RA is applied to the anterior margin, a 6-digit, double posterior limb develops instead of the normal 3-digit one (Tickle et al. 1982; Summerbell, 1983). RA is present endogenously in the normal chick limb bud and is concentrated 2.5-fold on the posterior side compared to the anterior side (Thaller and Eichele, 1987). This is precisely the behaviour we would expect if RA was the morphogen released by the zone of polarizing activity, the region on the posterior margin of the limb bud that controls the specification of pattern across the anteroposterior axis of the limb bud (Tickle et al. 1975). In the regenerating amphibian limb, RA causes respecification not only in the anteroposterior axis (Kim and Stocum, 1986; Maden, 1983), but also in the proximodistal axis (Maden, 1982) and the dorsoventral axis (Ludolph et al. 1990). RA is also present endogenously in the blastema of the regenerating limb (M. Maden, N. Watson and D. Summerbell, unpublished data). Thus RA may be the morphogen responsible for specifying axial information in the developing and regenerating limb.

In the early nervous system of the Xenopus embryo, RA administration causes an anteroposterior transformation whereby the forebrain and midbrain are reduced or absent and the hindbrain and spinal cord are correspondingly exaggerated (Durston et al. 1989). RA is also present endogenously in the whole Xenopus embryo at these stages thus fulfilling the two criteria listed in the first paragraph that suggest a role for RA in the specification of pattern in the early nervous system.

Inside the cell, RA interacts with two classes of protein: (1) cellular retinoic acid-binding protein
crested cells. A similar spectrum of CRABP-containing walls of the developing gut and as a population of dorsal late beside the dorsal aorta. They also appeared in the spreading ventrally through the sclerotome to accumulate individual CRABP-immunoreactive cells were seen outside the neural tube, subsequently becoming the neurons of the commissural system (Maden et al. 1989a). Outside the neural tube, several suggestions have been made as to the function of CRABP. It may be involved in the metabolism of RA, it may transport RA from its point of entry into the cell to the nucleus where the RARs are located thereby defining populations of cells that are RA-responsive (Maden et al. 1989a) or it may act as a buffer regulating the amount of RA available to the RARs and generating concentration gradients of RA (Maden et al. 1988; Smith et al. 1989).

One obvious way to begin investigations into the role of CRABP is to examine its distribution during development. This may also provide us with a more precise indication than that available by HPLC as to which tissues in the embryo utilise RA. Such an analysis with the RARy mRNA, for example, has shown that it is present in the branchial arches, limb buds, differentiating cartilages and keratinising epithelia of mouse embryos, suggesting a role for this receptor in cartilage and epithelial differentiation (Ruberte et al. 1990). Our previous experiments on the distribution of CRABP by immunocytochemistry in the chick embryo have identified two systems with interesting patterns of immunoreactivity, the limb bud and the nervous system i.e. the very systems that are RA responsive.

In the chick limb bud, CRABP is concentrated in the progress zone at the distal tip of the bud. Across the anteroposterior axis of the progress zone, CRABP is concentrated at the anterior margin, producing a gradient of opposite polarity to its ligand (Maden et al. 1988). In the mouse limb bud, an analysis of CRABP mRNA distribution by in situ hybridisation produced a similar result (Dollé et al. 1989). Dollé et al. also examined the distribution of RAR transcripts and found that RARα and RARy transcripts were uniform and there were no RARβ transcripts in the early limb bud thereby emphasising the importance of CRABP in the morphogenesis of pattern in the limb.

In the nervous system of the chick embryo, we have previously shown that CRABP immunoreactivity appears in the neuroblasts of the mantle layer, which subsequently become the neurons of the commissural system (Maden et al. 1989b). Outside the neural tube, individual CRABP-immunoreactive cells were seen spreading ventrally through the sclerotome to accumulate beside the dorsal aorta. They also appeared in the walls of the developing gut and as a population of dorsal root ganglion cells. We suggested that these were neural crest cells. A similar spectrum of CRABP-containing cells have been observed in the mouse embryonic nervous system both by in situ hybridisation (Perez-Castro et al. 1989) and immunocytochemistry (Maden et al. 1990).

In the work reported here, we examine in detail the contention that neural crest cells contain high levels of CRABP by comparing CRABP and HNK-1 patterns of immunoreactivity in the early chick embryo. HNK-1 is a monoclonal antibody that reacts with migrating neural crest cells (Vincent et al. 1983; Vincent and Thiery, 1984) and its distribution in the trunk of the chick embryo has been well characterised (Rickmann et al. 1985; Bronner-Fraser, 1986). We show that CRABP is indeed present in neural crest cells and their derivatives such as the frontonasal mass, branchial arches and cephalic ganglia. We have also investigated the time of first appearance of CRABP immunoreactivity in the neural tube because of the effects that RA has on the early nervous system (see above). We find a fascinating pattern of labelling, which begins in the hindbrain just after neural tube closure and produces a distinct striping array in the rhombomeres. The rhombomeres are currently arousing great interest as the sites of discrete homeobox gene localisations (Wilkinson et al. 1989) and homeobox gene expression, at least in cultured cells, is responsive to RA. Therefore we have also compared this pattern of CRABP immunoreactivity with that of the chicken Hox-2.9 gene using in situ hybridisation. We report that Hox-2.9 has a similar pattern of labelling in the rhombomeres. These results suggest that there may be a relationship between RA, CRABP and homeobox genes in the establishment of pattern in the nervous system, notably the hindbrain.

Materials and methods

Fertile chicken eggs were obtained from a mixed local flock (Needle Farm), incubated, and staged according to Lillie (1952). Embryos at stages 4–23 were fixed for 3 h in Perfix (Fisher Scientific, New Jersey) for immunocytochemistry, dehydrated, wax embedded and sectioned at 7 μm. CRABP was immunolocalised with an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to a sequence from bovine CRABP (Eriksson et al. 1987). The characteristics and specificity of this antipeptide antibody has been established and it has been used as an immunocytochemical reagent to localise CRABP in rat testis (Eriksson et al. 1987). We first confirmed that the antibody was appropriate for use with chicks by performing a Western blot. Whole chick embryos were homogenised in solubilisation buffer for SDS–polyacrylamide gel electrophoresis according to Laemmli (1970) and centrifuged to remove debris. Aliquots of the extract were run on a 12.5 % SDS–polyacrylamide gel and the proteins transferred to nitrocellulose paper. The immunoreactivity was visualised using the avidin–biotinylated peroxidase technique with a kit from Vector Laboratories (Peterborough, UK). The Western blot revealed that the antibody reacted with a single band of approximate 16×10^3 (Fig. 1), the expected molecular weight of CRABP.

Both HNK-1 (a gift of Dr Claudio Stern, University of Oxford) and CRABP antibodies were used on sections at a
CRABP, rhombomeres and neural crest

Results

Stage 9, 7 somites

This is the earliest stage at which CRABP was detected. Serial sections of several embryos revealed that fewer than 10 cells per embryo were immunoreactive and these were all located in the neural tube at the level of somites 1–3 (Figs 2A, 3). Since the neural folds had only just fused at the level of the mesencephalon/anterior rhombencephalon at stage 8, about 3 h earlier (Lillie, 1952), the appearance of CRABP-positive neuroblasts is a very early event in the development of the neural tube. The first 5 somites are located beside the posterior half of the rhombencephalon, thus conveniently marking the location of these early staining cells.

No other CRABP-positive cells were detected in the embryo either in the primitive streak, epiblast or mesoderm.

HNK-1-positive cells in the embryo were detected only in a small patch dorsal to the mesencephalon. This is precisely the stage and location (junction of...
cells are immunoreactive. An additional area of
packed in the space between the developing optic lobes
lic mesenchyme by migrating in streams and became
first time in these embryos, in the anterior mesencepha-
immunoreactivity in the neural tube appeared for the
serial sections are added together and plotted on a
differentiation proceeded (see Figs 8, 10, 12). In Fig. 4
or neural tube was immunoreactive, only those towards
the pial surface and this peripheral location remained as
and (3) in the anterior mesencephalon both dorsally
located in the cephalic mesenchyme at the anterior end
of the embryo in between the optic vesicle and the
ectoderm. Further posterior, neural crest cells
and the ectoderm. Further posterior, neural crest cells
could now be seen leaving the spinal cord by the ventral
route, between the neural tube and the somite as has
been described before for trunk neural crest cells
(Rickmann et al. 1985; Bronner-Fraser, 1986).

Stage 13, 18–20 somites
By now the pattern of CRABP immunoreactivity in the
neural tube had become well-established in three
locations (Fig. 2D): (1) in the developing neural tube
from the level of 6–8 somites behind the last-formed
somite through the posterior rhombencephalon up to
rhombomere 6; (2) in a single stripe in rhombomere 4
and (3) in the anterior mesencephalon both dorsally
and ventrally (Fig. 2D).

For the first time, CRABP-positive cells in the
mesenchyme were observed at this stage. These were
located in the cephalic mesenchyme at the anterior end
of the embryo in between the optic vesicle and the
ectoderm and dorsally above the prosencephalon
(Fig. 2D). What was most noticeable about these cells
is that they were indistinguishable in terms of location
from the early neural crest cells that populated the same
area around the developing forebrain at stage 12. It is
likely therefore that these CRABP-positive mesenchy-
mal cells are a subpopulation of neural crest cells that
have begun the synthesis of the retinoic acid-binding
protein.

Stage 10, 9–11 somites
Within the space of 3–4 h, the numbers of cells in the
neural tube that had CRABP immunoreactivity
increased markedly. The area over which they were
localised had now spread caudally to include the neural
tube adjacent to the first 5 somites (Fig. 2B) thus
confirming that the first neuroblasts to show this
phenotype were in the future rhombencephalon.
Outside the neural tube, there were no immunoreactive
cells either in the somites, primitive streak, notochord,
invaginating mesoderm etc.

The location of these CRABP neuroblasts within the
neural tube is of interest. In cross section, they were
excluded from the extreme dorsal or ventral poles of the
neural tube and were always located on the pial surface
rather than the ventricular surface (can be seen in
Fig. 3), although occasionally immunoreactivity was
present in a cell that extended across the whole width of
the neural tube. In the anteroposterior dimension, the
first cells to become labelled were located adjacent to
somites 6–8 behind the last-formed somite. These
principles were apparent throughout this study as the
embryos elongated and added somites at their posterior
ends.

HNK-1-positive cells could be seen in the cephalic
mesenchyme adjacent to the developing mesencepha-
lon migrating out from the neural tube in two distinct
streams of cells.

Stage 11, 12–15 somites
This was the first stage at which recognisable rhombo-
meres, the series of swellings in the rhombencephalon
were seen. There are 8 rhombomeres in all, but at this
early stage only 5 (numbers 2–6) could be delineated as
they develop over a period of time from stages 9–12
(Vaage, 1969; Fraser et al. 1990; Lumsden, 1990).
The numbering of individual rhombomeres can be assigned
by reference to the otic vesicle. What was immediately
obvious was that when sections were scored for the
presence of CRABP immunoreactivity only certain
rhombomeres contained positive neuroblasts (Figs 2C,
4). Thus rhombomeres 2, 3 and 5 were CRABP
negative and rhombomeres 4, 6 and continuing into the
anterior spinal cord were CRABP positive (Fig. 4). As
can be seen in Fig. 4, not every cell in the rhombomeres
or neural tube was immunoreactive, only those towards
the pial surface and this peripheral location remained as
differentiation proceeded (see Figs 8, 10, 12). In Fig. 4
only 7 cells are labelled in rhombomere 4, but when all
serial sections are added together and plotted on a
representative section, as in Fig. 2, a large number of
cells are immunoreactive. An additional area of
immunoreactivity in the neural tube appeared for the
first time in these embryos, in the anterior mesencepha-
lon (Fig. 2C).

HNK-1-positive cells continued to invade the cepha-
lic mesenchyme by migrating in streams and became
packed in the space between the developing optic lobes
and the ectoderm. Further posterior, neural crest cells
could now be seen leaving the spinal cord by the ventral
route, between the neural tube and the somite as has
been described before for trunk neural crest cells
(Rickmann et al. 1985; Bronner-Fraser, 1986).
Fig. 9–12. Sections of various staged chick embryos reacted with anti-CRABP antibody to show areas of immunoreactivity visualised as a brown reaction product.

Fig. 9. Sagittal section through the branchial arches of a stage 19 embryo. The maxillary arch (m) and arches 1–4 are marked. CRABP-positive cells are clearly peripheral within each arch and there is considerable variation in intensity of staining between the arches. Arch 1 is the most intense, followed by arches 3 and 4 with the maxillary process and arch 2 the least intense. Also apparent are occasional CRABP-positive cells, likely to be Schwann cells, in the developing cephalic nerves (arrows). Bar=100 μm.

Fig. 10. Transverse section through the rhombencephalon at stage 19 to show that the CRABP-positive neurons are in the mantle layer. Bar=100 μm.

Fig. 11. High-power view of the most anterior tip of a stage 21 embryo showing the absence of label in the telencephalon (t), the presence of CRABP-positive cells in the mesenchyme of the frontonasal mass (fnm) and the absence of label in the epidermis (e). Bar=25 μm.

Fig. 12. Low-power view of a sagittal section through the cephalic region of a stage 21 embryo to show the areas of immunoreactivity (as summarised in Fig. 2F). These CRABP-positive areas are: rhombencephalon (r), roof of the mesencephalon (m), frontonasal mass (f), branchial arches (a), otic vesicle (o). Note that the entire prosencephalon is unlabelled. Bar=25 μm.
HNK-1-positive cells were by this stage widely distributed throughout the embryo in well-characterised locations, namely: mesenchyme surrounding the fore- and midbrain; the region of the future facial primordia and branchial arches, although discrete arches were not yet evident; the facial and glossopharyngeal ganglia extending ventrally from the rhombencephalon; the developing heart and gut; the posterior half of the otic vesicle; and at the level of the somites, in the intersomitic clefts and in the anterior half of each somite.

**Stages 14–16**

During these stages there were several changes in CRABP immunoreactivity both in the neural tube and the mesenchyme. In the anterior neural tube, the area of labelled cells expanded posteriorly from the mesencephalon to encompass rhombomeres 1 and 2, thus changing the pattern from one labelled stripe (Fig. 2C, D) to two unlabelled stripes in rhombomeres 3 and 5 (Fig. 2E). The prosencephalon remained unlabelled.

In the mesenchyme, labelling of the most anterior cells surrounding the developing eye and telencephalon intensified. These were in the same location as the first HNK-1 cells to be labelled and, since the HNK-1 immunoreactivity in this area had now ceased, it seemed that CRABP was switched on in these cells when HNK-1 was switched off. Immunoreactivity also appeared for the first time in the branchial arches (Figs 2E, 5), again in cells that had switched off HNK-1. This branchial arch mesenchyme was clearly located peripherally (Figs 6, 8) being displaced by the cranial nerves and aortic arches. CRABP-positive staining also appeared in the otic vesicle (Fig. 5), as HNK-1 reactivity had done one stage earlier. In the region of the somites at stage 14, CRABP-positive cells could be seen in the anterior half of each somite and the intensity of this labelling increased over subsequent stages (see Fig. 7). These cells were thus likely to be neural crest migrating through the somites exactly as had been seen at earlier stages for HNK-1-positive cells. Lastly, occasional labelled cells began to appear in the cephalic ganglia (see Fig. 9).

The patterns of HNK-1 staining in cephalic regions over these stages showed an interesting complementarity to the CRABP staining. For example, in the branchial arch mesenchyme, the initial HNK-1 immunoreactivity that was noted at stage 13 had by now disappeared to be replaced by CRABP labelling. Thus HNK-1 cells could be seen approaching the arches, but within the arches themselves there was no labelling whereas those same arches labelled intensely with CRABP (Figs 6, 8). Also, the cells surrounding the optic vesicle and telencephalon had now ceased to be HNK-1-positive and had become CRABP-positive instead.

**Stages 17–23**

During the final period of development that we studied, the patterns of immunoreactivity described above intensified rather than changed dramatically. Fig. 12 shows an overall picture of CRABP immunoreactivity in the cephalic region and Fig. 2F a summary diagram.

The two gaps in CRABP immunoreactivity in the rhombencephalon gradually filled in so that labelling of these neuroblasts was continuous from the mesencephalon, through the rhombencephalon all the way to the end of the neural tube (Figs 8, 12). The prosencephalon remained unlabelled as it had done throughout development (see Fig. 12). In a cross section of the rhombencephalon, the lateral and ventral neurons of the mantle layer were the immunoreactive ones (Fig. 10). In the spinal cord, these neurons became those of the commissural system as we have previously described (Maden et al. 1989b).

The mesenchyme of the frontonasal mass, maxillary prominence and branchial arches remained intensely CRABP positive. Fig. 11 shows the immunoreactive frontonasal mass mesenchyme contrasting with the unstained diencephalon and the epidermis. Two other points are worth emphasising about the staining patterns of the arches. First it was specific to arch and facial mesenchyme, that is, the mesenchyme outside the developing arches or the mesenchyme not immediately underneath the facial epithelium was unstained. Second there was a considerable difference in the intensity of staining between different arches. The maxillary prominence and arch 2 were the faintest in CRABP-staining intensity, the frontonasal mesenchyme and arches 3 and 4 had intermediate intensity and arch 1 the strongest (Fig. 9).

The three other areas of the embryo that first showed CRABP immunoreactivity at earlier stages changed little during this period. The otic vesicle remained labelled, occasional labelled cells were seen in the cranial nerves (Fig. 9) and the immunoreactive cells migrating through the anterior halves of the somites became clearer (Fig. 7).

**Expression of Hox-2.9 gene**

The distribution of CRABP immunoreactivity in the rhombomeres immediately brings to mind the expression patterns of Hox-2 genes in the same structures in the mouse (Wilkinson et al. 1989). To examine similarities in the chick we have, as an initial experiment, looked at the distribution of the chick Hox-2.9 mRNA by in situ hybridisation, in the absence of an antibody to the Hox-2.9 protein.

In stage 9–10 embryos, the Hox-2.9 gene is expressed in a continuous domain along the neural tube extending into the hindbrain in a region coincident with that of CRABP. The anterior boundary of this domain is rhombomere 4. At stage 14–15 expression of Hox-2.9 is clearly seen in rhombomere 4, but there is now a gap in the domain of expression posterior to rhombomere 4 as shown in Fig. 13. Expression in this posterior domain extends through the anterior neural tube up to the region between rhombomeres 6 and 7 and appears to correspond to the boundary between these rhombomeres. However, as seen in Fig. 13 B–C on the right side of the neural tube some expression does appear to extend into rhombomere 6. The relevance of the
Fig. 13. Hox-2.9 homeobox expression in stage 14–15 chick embryos. (A) Dark-field photomicrograph of in situ hybridisation with the Hox-2.9 probe illustrating a strong stripe of expression in the hindbrain and a further domain of expression extending from the base of the hindbrain caudally along the neural tube. Bar=250 μm. (B) High-power bright-field and dark-field (C) micrographs showing the stripe of expression is segmentally restricted to rhombomere 4. The caudal domain of expression on the left side of the neural tube maps to the boundary between rhombomere 6 and 7, while on the right side it extends into the middle of rhombomere 6. (r) rhombomere, (ov) otic vesicle. Bars=60 μm.

association between CRABP and Hox genes is considered further below.

Discussion

We have described here the distribution of cellular retinoic acid-binding protein (CRABP), the protein to which retinoic acid (RA) specifically binds in the cytoplasm of cells, and compared it with the distribution of HNK-1, a monoclonal antibody that recognises migrating neural crest cells, at a full range of stages of early chick development. We also report the expression pattern of the chicken Hox-2.9 gene.

We are interested in CRABP because it may indicate those systems in development that use RA as a morphogen. The developing chick limb bud is one such case (Thaller and Eichele, 1987) and on the basis of the results reported here we propose that the generation of pattern in the early nervous system is another case. We used HNK-1 staining because our previous studies had suggested that CRABP was present in neural crest cells (Maden et al. 1989) and we wished to see if CRABP and HNK-1 stained the same populations of cells.

After examining serial sections of embryos up to stage 23, it was apparent that CRABP was present in only two areas of the embryo, the neural tube and a subpopulation of neural crest and its derivatives (the additional area is the limb bud – see Maden et al. 1988).

CRABP in the neural crest

From the comparative patterns of immunoreactivity of CRABP and HNK-1, it was clear that the neural crest does indeed contain high levels of CRABP as we had previously suggested (Maden et al. 1989). However, CRABP immunoreactivity appeared in the crest some time after HNK-1 immunoreactivity. In the trunk, for example, HNK-1-positive cells were present in the anterior halves of the somites at stage 13, but CRABP-positive cells in the same location were not seen until stages 14–16. This unique behaviour of trunk neural crest cells in migrating through the anterior half of each somite and the same behaviour being elicited by CRABP-positive cells confirms that migrating neural crest does contain CRABP.

In cephalic regions, however, the behaviour of these two antibodies was slightly different. CRABP did not appear until after HNK-1 staining had disappeared, the two antibodies thus showing a complementary pattern. This was particularly apparent in the branchial arch mesenchyme in which HNK-1 staining disappeared as the neural crest entered the arches to be replaced by intense CRABP staining. Thus rather than playing a role in migration, RA is more likely to be involved in the differentiation of the neural crest upon arrival at its destination. Our previous observations in the trunk of the chick embryo (Maden et al. 1989) showing CRABP immunoreactivity in a population of dorsal root ganglion cells, in the sympathetic ganglia and in the walls of the developing gut, support this contention.

CRABP in the neural tube

The first neuroblasts to become CRABP positive
appeared at stage 9 in the rhombencephalon, very soon after closure of the neural folds (at stage 8). These neuroblasts were situated within the mantle layer of the neural tube although an occasional positive cell could be seen stretching from the pial to the ventricular surface. This location at the pial surface is the same as that seen with N-CAM and the $68 \times 10^3 M_r$ neurofilament protein labelling at later stages in the rhombencephalon (Lumsden and Keynes, 1989).

As the neural tube continued to develop and somites were added in a posterior direction, CRABP immunoreactivity also spread posteriorly within the neural tube to remain about 6–8 somites behind the last formed somite. By stage 11, a remarkable pattern of labelling had appeared in the newly formed rhombomeres such that a single stripe was created. Immunoreactivity was present up to and including rhombomere 6, it was absent in rhombomere 5, present in rhombomere 4 and absent again in the rest of the hindbrain (Fig. 2C, D). This pattern remained until stages 14–16 by which time rhombomeres 1 and 2 had become labelled thus creating two unlabelled stripes (Fig. 2E). Following this the entire hindbrain became labelled and the striping pattern was lost (Fig. 2F). Over this period, the mesencephalon was also CRABP positive, especially the roof, whereas the prosencephalon remained unlabelled.

CRABP immunoreactivity is thus one of the earliest markers of differentiation yet described within the rhombomeres, the homeobox genes being the other (see below). It precedes the appearance of neurofilament immunoreactivity of reticular neurons which appears in the neurons of even-numbered rhombomeres at stage 11/12 (Lumsden, 1990). CRABP appears during the period that the rhombomeric ‘compartments’ are established (Fraser et al. 1990) and its sequence of appearance seems to be the same as the sequence of differentiation, that is, even numbered rhombomeres before odd numbered ones (Lumsden and Keynes, 1989).

**CRABP and RA localisation**

As described in the Introduction, one reason for examining the distribution of CRABP in the embryo is to provide an indication of which structures use RA in their development. Since CRABP specifically binds RA, it may be valid to assume that cells containing higher levels of CRABP contain higher levels of RA than cells that do not contain CRABP. Because of the impossibility of collecting large amounts of embryonic material, this information would not be available by current HPLC techniques. But is this assumption valid?

Evidence that this is valid has recently been obtained by injection of pregnant mice with $^3H$-RA (Dencker et al. 1987) or $^{14}C$-Ro 13–6298, a RA analogue (Dencker et al. 1990). Radioactivity localises to specific regions of the mouse embryo. These regions are: early neural crest migrating from the neural folds, the midbrain roof, the hindbrain, the frontonasal mass, the branchial arches (particularly the periphery rather than the core region), dorsal root ganglia and the mantle layer of the neural tube. These are exactly the regions that contain high levels of CRABP both in the chick (Maden et al. 1989b; these results) and the mouse (Perez-Castro et al. 1989; Vaessen et al. 1989; Dencker et al. 1990; Maden et al. 1990) embryo. Thus the coincidence between high RA and high CRABP in discrete regions of the embryo is experimentally verifiable.

**CRABP and the effects of RA on chick embryos**

It would be a valuable exercise to compare the pattern of CRABP expression with the effects of teratogenic doses of RA on the embryo to see if the two are related. Compared to the mammalian embryo, however, few teratogenicity studies have been conducted on the chick embryo. Nevertheless it seems clear that neural tube development is arrested and cardiovascular, limb and beak defects appear (Jelinek and Kistler, 1981). It may be no coincidence that the cells of each of these systems contain high levels of CRABP. Interestingly, during the development of the face only the upper beak is affected by RA, the lower beak being immune from its teratogenic action (Tamarin et al. 1984) and this effect is mediated via the mesenchyme (Wedden, 1987). As we have shown here, the components that go to make up the face and beak show differing intensities of CRABP staining, which may be related to their differing susceptibility to RA. For example, only the mesenchyme of the frontonasal mass and branchial arches is CRABP-positive, the epidermis is negative and it is only the mesenchyme that is affected by RA. However, the upper beak/lower beak discrepancy is more difficult to explain in these terms. The upper beak develops from the frontonasal mass and maxillary process whereas the lower beak develops from the first arch. But the first arch shows the most intense CRABP staining which, according to the above scheme, should make it more susceptible to teratogenic doses of RA. So there must be other components such as the RARs and RXRs involved in the teratogenicity of RA.

**Relationship between CRABP and Hox genes**

The dynamic spatial and temporal patterning of CRABP that we have described in the rhombomeres is reminiscent of the patterns of activity of the Hox-2 genes, which show segment-restricted expression in the hindbrain (Wilkinson et al. 1989). Recently we have found that the Hox-2 genes are also expressed in cranial neural crest cells, displaying sharp boundaries of expression in crest from different arches (Hunt, Wilkinson and Krumlauf, in preparation).

One obvious question to ask therefore is whether any of these Hox and CRABP patterns are coincident. Previous in situ hybridisations in the hindbrain with homeobox probes have been performed on the mouse and as part of a similar analysis in the chick with the homologous chick probes we examined here the distribution of Hox-2.9. It is important to note that, in the absence of a Hox-2.9 antibody, we were comparing mRNA accumulation with protein accumulation, the two not necessarily being comparable. Nevertheless, the patterns of Hox-2.9 and CRABP were very similar
between stages 9 and 15. Initially, a uniform domain of expression extending into the hindbrain up to the anterior boundary of rhombomere 4 was observed for both genes. This was followed by a regression or drop in expression in rhombomere 5. These temporal changes in the establishment of segment-restricted patterns are of particular interest in view of the fact that they are displayed by two different kinds of genes, and may reflect events in the ordering and specification of different rhombomeres. The only difference between CRABP and Hox-2.9 in the neural tube at stage 14–15 occurred in rhombomere 6 where CRABP was expressed but Hox-2.9 was only faintly detected in part of rhombomere 6 on one side of the neural tube (Fig. 13 B, C). At later stages the pattern of CRABP continued to change while that of Hox-2.9 remained the same at least until stage 19.

CRABP and Hox-2 mRNA also co-localise in the mantle layer of the mouse spinal cord in the region forming the commissural and association neurons (Graham et al. 1990; Maden et al. 1990). The only other analysis of the codistribution of Hox genes and retinoid-binding proteins concerns Hox-2.5 and cellular retinol-binding protein (CRBP) in the transverse axis of the mouse spinal cord (Graham et al. 1990). In this case we found that these two occupied reciprocal and adjacent domains with Hox-2.5 being dorsal and CRBP ventral. Understanding the significance of these observations must await a more complete analysis of the role of the retinoid binding proteins and the role of the retinoid acid receptors (see Introduction) in relation to Hox gene activity. Nevertheless they suggest that other such coincidence should be sought, for example, between CRABP, RAR, RXR and Hox genes.

**RA and pattern formation in the hindbrain**

When several pieces of evidence are brought together it appears likely that RA may play a role in the development of pattern in the hindbrain. First, RA causes anteroposterior transformations in the *Xenopus* neural tube whereby the forebrain and midbrain are reduced and the hindbrain and spinal cord are correspondingly exaggerated (Durston et al. 1989). In fact, alterations in specific cranial ganglia associated with the rhombomeres can be detected in *Xenopus* embryos at certain concentrations of exogenously administered RA (N.Papalopoulou and R.Krumlauf, unpublished results). Second, in mouse F9 teratocarcinoma cells all of the genes in the Hox-2 complex are rapidly inducible by RA. The relative responsiveness of any particular gene varies with RA concentration and with time and there is a linear correlation between their ability to respond to RA and their organisation in the Hox-2 cluster (Simeone et al. 1990; Papalopoulou et al. 1990). This correlation between homeobox genes and RA responsiveness was also observed for several *Xenopus* Hox genes when whole embryos were exposed to brief treatment with RA (Papalopoulou et al. 1990, in preparation). Finally from the results of this study the distribution of CRABP and Hox-2.9 are coincident in the hindbrain. Thus it is possible that RA via CRABP and the RARs and RXRs could also be used to establish gradients of homeobox gene expression, thereby imparting identity upon previously uniform segments of the developing central and peripheral nervous system.

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**References**


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