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The role of retinoid-binding proteins in the generation of pattern in the developing limb, the regenerating limb and the nervous system

M. MADEN[1], D. E. ONG[2], D. SUMMERBELL[1] and F. CHYTIL[2]

[1]*National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA UK*
[2]*Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee, USA*

Summary

We summarise existing data and describe new information on the levels and distribution of cellular retinoic acid-binding protein (CRABP) and cellular retinol-binding protein (CRBP) in the regenerating axolotl limb, the developing chick limb bud and the nervous system of the chick embryo in the light of the known morphogenetic effects of retinoids on these systems. In the regenerating limb, levels of CRABP rise 3- to 4-fold during regeneration, peaking at the time when retinoic acid (RA) is most effective at causing pattern duplications. The levels of CRBP are low. The potency of various retinoids in causing pattern respecification correlates well with the ability of these compounds to bind to CRABP. In the chick limb bud, the levels of CRABP are high and the levels of CRBP are low. Again the binding of various retinoids to CRABP correlates well with their ability to cause pattern duplications. By immunocytochemistry, we show that CRABP is present at high levels in the progress zone of the limb bud and is distributed across the anteroposterior axis in a gradient with the high point at the anterior margin. In the chick embryo, CRABP levels are high and CRBP levels are low. By immunocytochemistry, CRABP is localised primarily to the developing nervous system, labelling cells and axons in the mantle layer of the neural tube. These become the neurons of the commissural system. Also sensory axons label intensely with CRABP whereas motor axons do not and in the mixed nerves at the brachial plexus sensory and motor components can be distinguished on this basis. In the neural tube, CRBP only stains the ventral floor plate. Since the ventral floor plate may be a source of chemoattractant for commissural axons, we suggest on the basis of these staining patterns that RA may fulfill this role and thus be involved morphogenetically in the developing nervous system.

Key words: retinoic acid-binding protein, retinol-binding protein, retinoic acid, retinol, chick limb bud, chick embryo, pattern formation, axolotl, limb regeneration, neural tube, sensory nerves, ventral floor plate, nervous system.

Introduction

Vitamin A has been the subject of extensive research since its discovery as a fat-soluble vitamin in the first part of this century. Because of the importance of vitamin A in nutrition, its involvement in vision, the maintenance of epithelial differentiation and spermatogenesis was soon established. Any deficiency or excess of vitamin A in the diet leads to a disturbance in these tissues and organs. Retinol is the parent vitamin A molecule and the many derivatives of this structure are known collectively as retinoids.

Uptake of vitamin A by the body

β-carotene, a plant pigment, and retinyl esters in animal tissues such as liver, oils and egg yolk are the major dietary sources of vitamin A. These esters are hydrolysed in the gut to retinol which is absorbed into the lining and reesterified. The transport of retinyl esters from the small intestine is accomplished *via* chylomicrons released into the lymph and these esters are stored in the liver. When needed by the tissues of the body the esters are hydrolysed to retinol and then transported in the blood bound to serum retinol-binding protein (RBP), a 21K ($K=10^3 M_r$) protein (Ong, 1985). Cellular uptake of retinol is dependent upon a specific membrane receptor that recognises RBP and transfer to the cytoplasmic retinol-binding protein involves a further cycle of esterification (Otonello *et al.* 1987). Thus cells receive vitamin A in the form of retinol.

Cellular retinoid-binding proteins

Cellular retinol-binding protein (CRBP) is a 14.6K protein present in the cytosol of a wide variety of cell types (Chytil and Ong, 1984) and as just described is responsible for receiving the retinol delivered to the cell. It has a single binding site with a high degree of specificity for retinol which is its endogenous ligand

(Saari *et al.* 1982). CRBP has been shown to be capable of transferring retinol to specific binding sites in isolated rat nuclei or chromatin (Takase *et al.* 1979).

The other cytoplasmic binding protein widely distributed in cells is cellular retinoic-acid-binding protein (CRABP) which also has a relative molecular mass of 14.6×10^3 (Chytil and Ong, 1984). Like CRBP, CRABP has very specific binding properties and its endogenous ligand is RA (Saari *et al.* 1982). It too has been shown to transfer RA to specific binding sites in nuclei (Takase *et al.* 1986). The source of this retinoic acid (RA) is almost certainly oxidation of retinol as little or no RA is found in the diet. Thus if a cell needs RA it will synthesise it for itself (Napoli and Race, 1987), suggesting that, if RA is found in restricted regions of the embryo, it may be playing an important morphogenetic role.

Retinoic acid receptors

It was therefore assumed that the function of CRBP and CRABP was to behave like steroid hormones and deliver their ligands to the nucleus to produce a change in the pattern of gene activity (Chytil and Ong, 1984). However, upon sequencing, no DNA binding domains were found in these proteins and the situation was somewhat enlightened when a higher molecular weight nuclear protein, specific for RA, was detected in F9 cells (Daly and Redfern, 1987). Subsequently, two nuclear receptors with high affinity for RA were cloned on the basis of their homology to steroid receptors (Petkovich *et al.* 1987; Giguere *et al.* 1987; Brand *et al.* 1988; Benbrook *et al.* 1988). Thus it now seems likely that CRABP is a cytoplasmic protein which passes RA on to the nuclear retinoic acid receptors (RARs). In the absence of a retinol receptor, the function of CRBP remains uncertain.

Retinoids and the embryo

Until very recently the only information about retinoids and embryonic development came from nutritional studies in which pregnant mammals were fed on vitamin A-deficient diets or fed excess vitamin A. Embryos born to mothers raised on vitamin A-deficient diets, if they survive at all, have a variety of congenital abnormalities such as anophthalmia, microphthalmia, defects of the retina, cleft palate, hydrocephalus, malformed hind legs, cryptorchidism, cardiovascular malformations and urinogenital tract malformations (Kalter and Warkany, 1959). Retinoids are highly teratogenic and the embryonic defects produced under conditions of hypervitaminosis A are surprisingly similar to those described above, namely anophthalmia, microphthalmia, cleft palate, defects of the retina, hydrocephalus, spina bifida and limb defects (Kalter and Warkany, 1959).

Retinoids and the developing limb

Direct evidence for the role of retinoids in development must depend on their identification and quantification in individual organ systems within the embryo. The first such analysis by HPLC of the developing chick limb bud produced exciting results (Thaller and Eichele, 1987). Upon dissecting the limb bud into two, it was found that, whereas retinol was in equal amounts in the two parts, RA was enriched in the posterior part. They interpreted this result to mean that there is a gradient of RA across the anteroposterior (AP) axis of the limb bud with the high point on the posterior side.

The reason for looking at RA in the limb bud in particular was because this compound had been shown to have remarkable effects on pattern formation in the AP axis. When RA is applied locally to the anterior side of the limb bud, it causes mirror-image duplications in the AP axis such that 6-digit double posterior limbs develop instead of the normal 3-digit ones (Tickle *et al.* 1982; Summerbell, 1983). The behaviour of RA precisely mimics the effects of grafting the zone of polarizing activity (ZPA), a group of cells at the posterior margin, which organises pattern across the AP axis of the limb (Tickle *et al.* 1975). This coincidence led to the suggestion that RA may be the natural morphogen that the ZPA uses to generate pattern (Eichele *et al.* 1985) and the data on its endogenous distribution (Thaller and Eichele, 1987) provide powerful support for this notion.

Retinoids and limb regeneration

It had earlier been shown in the regenerating limbs of toads that, instead of regenerating just the foot after amputation through the shank, extra limb segments were produced if the animal was treated with retinyl palmitate (Niazi and Saxena, 1978). In newts and axolotls, the same specific effects on the proximodistal (PD) axis were observed such that complete limbs could be regenerated from amputations through the hand (Maden, 1982; 1983*a*; Thoms and Stocum, 1984). In addition to these effects on the PD axis, pattern duplication in the AP axis can also be demonstrated (Maden, 1983*b*; Kim and Stocum, 1986) as well as in the dorsoventral axis (Stocum, unpublished). In the light of these effects on each of the three cardinal limb axes, it is difficult to see how RA could be a morphogen in the way that it is believed to be in the chick limb bud. But we still need to know the biochemical and molecular mechanisms of action of RA in the cells of the regenerating limb because it is clearly acting on the endogenous pattern generating mechanisms.

CRABP and CRBP in the limb and embryo

As a first step towards understanding the mode of action of RA either as a morphogen or as a chemical that specifically disturbs pattern generating mechanisms, we describe here our recent experiments on the levels and distribution of CRABP and CRBP. In the first section, we consider the regenerating axolotl limb and the developing chick limb bud in the light of the effects of RA on the two systems. In the second section, we have examined other regions of the chick embryo for the presence of retinoid-binding proteins and find them to be most interestingly localised in the developing nervous system. Because of this distribution we suggest that RA is acting as a morphogen in the nervous system as well as the limb.

The regenerating amphibian limb

CRABP

The presence of retinoid-binding proteins can be detected in high-speed supernatants of homogenised tissues by a variety of means. We have used sucrose gradient centrifugation assays in which a fixed volume of protein preparation is incubated with [^{3}H]RA either in the presence or absence of a 100× molar excess of cold RA. The incubations are then dialysed to get rid of the excess radioactivity and spun on 5–20% sucrose gradients. If CRABP is present a peak of radioactivity appears at the point on the gradient which corresponds to its known molecular weight (approx. 16K) and if the binding is specific then there should be a drop in activity when excess cold RA is present (Fig. 1). By this means we determined that CRABP is present at low levels in some of the tissues of the larval axolotl, such as skin and muscle, and undetectable in liver and serum (Keeble and Maden, 1986). The latter two results were expected from data on mammals (Chytil and Ong, 1984) and confirmed that the assays were working and that axolotls did not have abnormal vitamin A metabolism.

When the level of CRABP in unamputated limbs and regeneration blastemas were compared a 3- to 4-fold rise was observed (Fig. 1; Table 1). This rise was maximal in the cone-stage blastema and as regeneration progressed so the level decreased to that found in unamputated limbs (Keeble and Maden, 1986). The early blastemal stage is also the period when exogenously administered RA has the most profound effect on pattern (Maden *et al.* 1985), a coincidence that suggests a role for CRABP in the mechanism of action of RA. When cone-stage blastemas were treated with a dose of RA sufficient to cause proximodistal duplications, no increase in CRABP could be detected so the levels of this protein do not seem to be regulated by the concentration of its ligand.

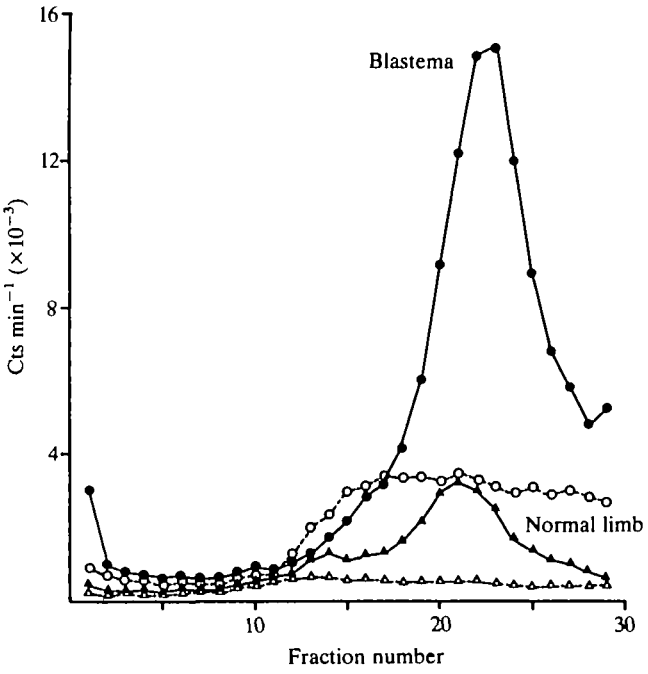


Fig. 1. Sucrose density gradient profile of axolotl limb and blastema cytosol to detect the presence of CRABP. Equivalent amounts of protein incubated with 66 nM-[^{3}H]all-*trans*-retinoic acid (NEN, specific activity 55 Ci mmol^{-1}) alone (●——●, ▲——▲) or in the presence of 100× M excess cold all-*trans*-retinoic acid (○---○, △---△). ●——●, ○---○ cytosol from cone stage regeneration blastemas. ▲——▲, ▲---▲ cytosol from normal, unamputated limbs. The drop in cts min^{-1} in the presence of cold RA is a measure of relative activity levels. The peak of activity is in the part of the gradient to which a myoglobin marker (17K) sediments.

Table 1. *Levels of CRABP and CRBP in various developing and regenerating tissues measured by sucrose density centrifugation assays*

Tissue	Concentration (fmoles per mg cytosolic protein) CRABP	CRBP
Axolotl unamputated limb	700±30	162±44
Axolotl regeneration blastema	2600±100	471±117
Chick limb bud	9100±1200	805±79
Mouse limb bud	9780±1780	1075±325
Chick embryo	6700±400	660±300

CRABP has also been detected in axolotl limbs by a different method, that of high-performance size-exclusion chromatography (McCormick *et al.* 1988). In this work the results obtained are, at first sight, somewhat different from those described above because no substantial difference in total CRABP was detected between unamputated and regenerating limbs. However, stage-dependent differences were detected in the levels of apo-CRABP (CRABP without any endogenous RA bound) and holo-CRABP (CRABP with RA bound). Apo-CRABP was highest at the blastemal stage and as regeneration proceeded it became saturated with endogenous RA such that only holo-CRABP could be detected at the 2-digit regenerate stage. Since the sucrose gradient method (Fig. 1) would be expected to measure apo-CRABP plus some fraction of the holo-CRABP due to ligand exchange, the two sets of data seem to show a similar stage-dependent rise and then decline in the availability of CRABP, implying a role for this molecule in limb regeneration (see below).

Competition and potency studies

In an effort to examine the role of CRABP in more detail, we have compared the ability of a range of retinoids to respecify the proximodistal pattern of the regenerate with their ability to bind to CRABP. If retinoids that do not affect pattern do not bind to CRABP and, conversely, those that do affect pattern do bind to CRABP then this would provide strong support for the involvement of CRABP in the mechanism of pattern respecification. We have tested a total of nine retinoids with different polar end groups or altered ring or side chain structures compared to the basic RA molecule (Keeble and Maden, 1989).

With regard to their effect on pattern formation, when administered locally in a silastin implant (see

Table 2. *Relative potencies (ability to respecify pattern) and binding affinities to CRABP (measured as IC_{50}) of nine different retinoids tested on axolotl regeneration blastemas and chick limb buds*

Retinoid	Structure	Axolotl		Chick	
		Potency	Affinity	Potency	Affinity
Retinoic acid		1	4×10^{-7}	1	2×10^{-7}
Ro 13-7410		100+	2×10^{-7}	2	1×10^{-7}
Arotinoid		10	1.2×10^{-6}	2	0
Ro 10-1670		0 1	$6\ 7\times10^{-7}$	0 7	1.8×10^{-6}
Etretinate		0	0	0	0
Retinal		0	1.4×10^{-5}	0	2.2×10^{-6}
Retinol		0	0	0	0
Retinyl acetate		0	0	0	0
Retinyl palmitate		0	0	0	0

Potencies are assessed relative to all-*trans*-retinoic acid Ro numbers refer to Hofmann-La Roche code numbers.

Maden *et al.* 1985) retinyl palmitate, retinyl acetate, retinol, retinal and etretinate were inactive (Table 2). Each of these compounds has an altered polar end group compared to the acid moiety of RA (see Table 2 for structural details), thus it is clear that this part of the molecule is crucial in determining biological activity. The compound Ro 10–1670, which has an acid end group like RA but an altered ring structure, has considerably less activity than RA. Arotinoid, which has an ester end group but 2 additional ring structures in the side chain, is 5–10× more potent than RA. Ro 13–7410, which has an acid end group and 2 additional ring structures in the side chain, is at least 100× more potent than RA (Table 2). Thus the addition of rings into the side chain of the RA molecule dramatically increases its potency at respecifying pattern.

Concerning their ability to bind to CRABP, similar principles emerged. Ro 13–7410 had twice the affinity for CRABP compared to RA, suggesting that the addition of rings into the side chain increased binding. Ro 10–1670 had only two thirds the affinity, suggesting that altering the parent ring structure decreased binding. Arotinoid and retinal had decreased affinity and retinol, retinyl acetate and retinyl palmitate had no affinity for CRABP, suggesting that the polar end group is important for binding.

Thus, in order to bind to CRABP, a retinoid needs an acid end group and such compounds are most effective in altering pattern. The addition of extra ring structres into the molecule increases binding and increases pattern respecification potency. These correlations imply a role for CRABP in pattern respecification. Arotinoid, however, does not strictly fit with these rules (Table 2) because its binding to CRABP is less avid than RA, but it is more potent than RA in respecifying pattern. This may be the exception that disproves the thesis or arotinoid may be metabolised by esterases in the limb to the more potent compound Ro 13–7410 and its potency at respecifying pattern thus exaggerated.

CRBP

The other retinoid-binding protein which is known to exist in mammalian tissues and is specific for retinol is CRBP. It is important to know whether this is present in the axolotl limb and during regeneration. By sucrose gradient assay CRBP was virtually undetectable in the unamputated limb, but clearly present in the cone-stage regeneration blastema (Fig. 2). However, the level was only one quarter of that of CRABP (Table 1).

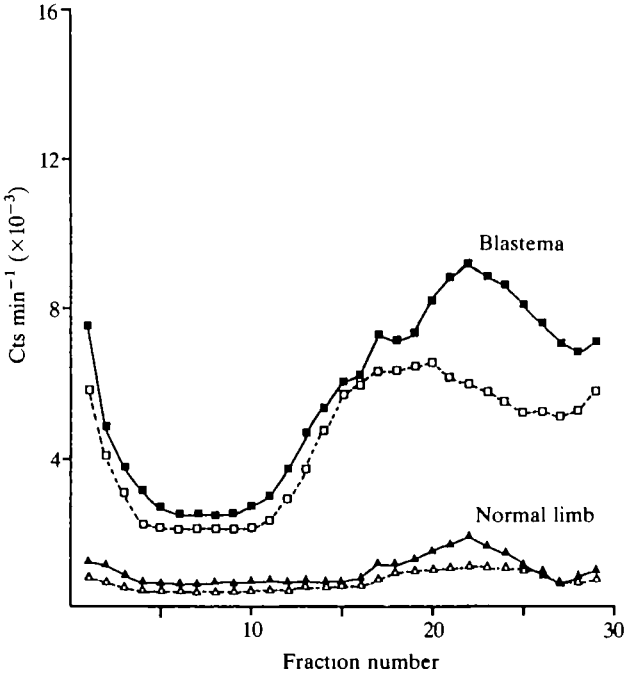


Fig. 2. Sucrose density gradient profile of axolotl limb and blastema cytosol to detect the presence of CRBP. Equivalent amounts of protein incubated with 66 nM-[^{3}H]all-*trans*-retinol (Amersham, specific activity 60 Ci mmol^{-1}) alone (■——■, ▲——▲) or in the presence of 500× M excess cold all-*trans*-retinol (□---□, △---△). ■——■, □---□ cytosol from cone-stage regeneration blastemas. ▲——▲, △---△ cytosol from normal, unamputated limbs. The drop in cts min^{-1} in the presence of cold retinol is a measure of relative activity levels. The peak of activity is in the part of the gradient to which a myoglobin marker (17K) sediments.

The role of CRBP in regeneration is not clear. The lower levels of this protein could imply a lesser significance. Indeed retinol applied locally does not cause pattern respecification (Keeble and Maden, 1989). When applied systemically, however, retinol is active (Maden, 1983), but this may be due to the metabolic

conversion of retinol to RA. Nevertheless, it would be unwise to rule out this molecule in the process of limb regeneration until more data such as its immunocytochemical distribution become available.

Speculations

The observations described above permit several interesting speculations about the mechanisms involved in pattern formation during limb regeneration. First, since RA is the endogenous ligand for CRABP (Saari *et al.* 1982) and CRABP levels rise and fall during regeneration it is highly likely that RA is involved in the process of limb regeneration. Whether it is a morphogen, as it is thought to be in the developing limb (Thaller and Eichele, 1987), is not at all clear. In the chick limb bud only the anteroposterior (AP) axis is affected by RA, but in the regenerating limb all three axes (PD, AP and DV) can be respecified (see Introduction). Classically, a Cartesian coordinate system such as this would be expected to be organised by gradients of three different morphogens (Wolpert, 1969) and it is difficult to see how only one could do the job.

Second, McCormick *et al.* (1988) have suggested from their data on apo- and holo-CRABP that the effects of exogenous RA on pattern respecification could be mediated by two different mechanisms. It was established early on in the analysis of retinoid effects on regenerating limbs that there was a stage dependency (Maden, 1983; Niazi *et al.* 1985; Maden *et al.* 1985). When RA is administered at early to midblastemal stages the effect is to proximalise the regenerate. At this stage, apo-CRABP is at its highest level and thus available for binding the exogenously administered RA which could then mediate proximalisation *via* an interaction with the genome. When RA is administered at later stages, by which time redifferentiation is already well under way (e.g. 2-digit stage) then only inhibitory effects such as missing phalanges or missing digits are produced. At these stages all the CRABP is in the form of holo-CRABP and thus the exogenous RA remains free and could directly inhibit biosynthetic processes such as cartilage formation (Hassell *et al.* 1978).

Thirdly, McCormick *et al.* (1988) also consider the possibility that a differing spatial distribution of apo- and holo-CRABP could explain the effects of RA on the anteroposterior axis of the regenerating limb. As described in the Introduction when double-anterior half limbs are treated with RA the regenerate is proximalised *and* mirror-imaged in the AP axis. RA thus induces posteriorisation of anterior cells. But when double-posterior half limbs are similarly treated regeneration is inhibited. This could be explained if the anterior region of the blastema was rich in apo-CRABP and thus exogenous RA could be bound, interact with the genome and posteriorise the cells. If CRABP in the posterior region were virtually all in the holo- form then exogenous RA could not be bound, would thereby remain free and thus inhibit biosynthetic process directly. Alternatively there may be an AP gradient of total CRABP with the high point on the anterior side as has been found in the chick limb bud (Maden *et al.* 1988).

Clearly, we need to find out a lot more about the precise cellular and spatial distribution of CRABP and CRBP in the regenerating limb. Immunocytochemistry would be extremely valuable as it has been in the chick limb bud (see below), although this technique is unlikely to provide a full description as it cannot, for example, distinguish between apo- and holo-CRABP. It will also be important to establish the distributional relationship between CRABP and the retinoic acid receptors (see Introduction) both within the cell and within the blastema during the process of limb regeneration.

The developing chick limb bud

CRABP

We found high levels of CRABP in the chick limb bud in sucrose gradient assays (Fig. 3, Table 1) which remained high at all the stages examined (Maden and Summerbell, 1986). The total specific binding capacity was estimated to be 14–28 pmoles mg^{-1} protein and the K_d 140–280 nM. This estimate of dissociation constant is at the upper end of the range of values from other tissues which varies from 2 nM for mouse limb buds (Kwarta *et al.* 1985) and 4.2 nM for rat testis (Ong and Chytil, 1978) to 318 nM for rat testis (Bonelli and

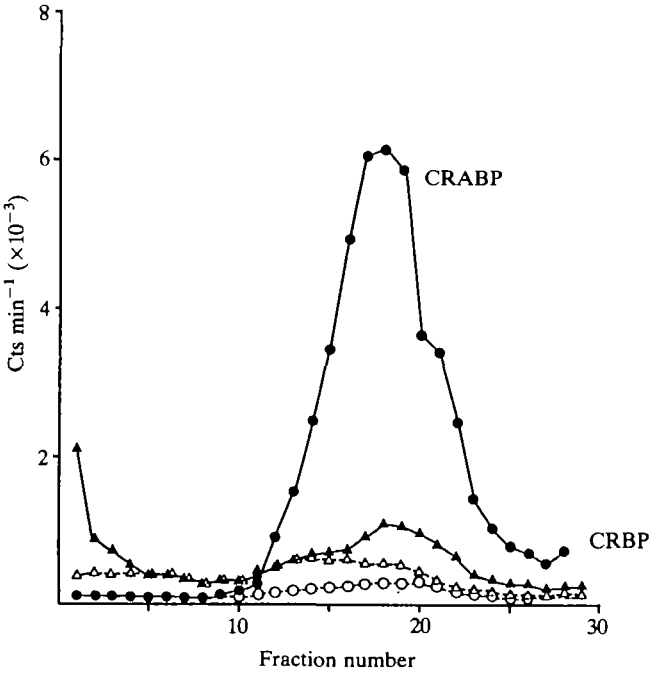


Fig. 3. Sucrose density gradient profiles of stage 24 chick limb bud cytosol to detect the relative levels of CRABP (●——●, ○---○) and CRBP (▲——▲, △---△). Equivalent amounts of protein incubated with: 66 nM-[^{3}H]all-*trans*-retinoic acid (●——●), [^{3}H]RA plus 100× M excess cold RA (○---○), 66 nM-[^{3}H]all-*trans*-retinol (▲——▲), [^{3}H]retinol plus 500× M excess cold retinol (△---△).

DeLuca, 1985) and 400 nm for human skin (Siegenthaler and Saurat, 1985).

Competition and potency studies

As described above for axolotl blastemal CRABP, we have examined the binding affinities of various retinoids for chick limb bud CRABP and compared this with the ability of these compounds to respecify pattern in the AP axis of the limb bud.

A similar sequence of affinities was found (Maden and Summerbell, 1986) indicating similar structural requirements for binding when compared to axolotl blastemal CRABP (Table 2). Thus Ro 13–7410 had twice the affinity for chick limb bud CRABP than did its natural ligand all-*trans*-RA. Ro 10–1670 was about 10× less effective at binding than RA and retinal was about 12× less effective. The remaining analogues – arotinoid, etretinate, retinol, retinyl palmitate and retinyl acetate – did not show any affinity for CRABP. Thus the primary structural requirement was an acid end group and the presence of two additional rings in the side chain increased binding affinity.

Regarding the effect of these retinoids on pattern formation, it is known that Ro 13–7410 is more potent than RA (Eichele *et al.* 1985) and we have confirmed that result (Table 2). Thus the two additional rings in the side chain increase potency as well as binding affinity. Arotinoid, which also has two additional rings, is nearly twice as potent as RA although it does not bind to CRABP. Ro 10–1670 is less potent than RA despite having an acid end group and does not bind to CRABP as well as RA. The remaining analogues – etretinate, retinol, retinal, retinyl palmitate and retinyl acetate – were inactive (Table 2). Thus the sequence of binding affinities is the same as the sequence of potencies with the exception of arotinoid, which is active but does not bind to CRABP. As discussed above for the axolotl data, it is possible that arotinoid is metabolised to Ro 13–7410 by endogenous esterases in the limb thus causing the potency score to be exaggerated. Alternatively arotinoid may be the exception that demonstrates that these compounds do not have to bind to CRABP to be biologically active. We are currently examining the metabolism of retinoids in the chick embryo in an attempt to solve this problem.

CRBP

In sucrose gradient assays CRBP is barely detectable in the chick limb (Fig. 3), having only about 1/10th of the level of CRABP (Table 1). We have failed to detect CRBP immunocytochemically in the limb bud (see below) and therefore presume that this protein does not play a significant role in the establishment of pattern in this system.

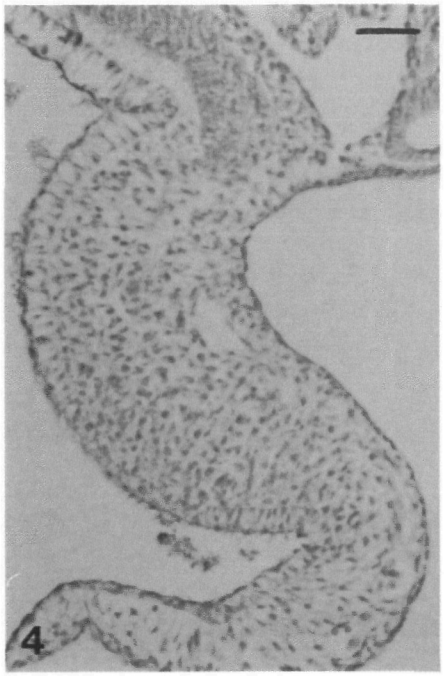
Immunocytochemistry

A great deal of valuable information has been obtained with the use of antibodies to these binding proteins. To look at the distribution of CRABP, we have used an affinity-purified rabbit polyclonal antibody made from rat CRABP. This antibody reacts with a single protein of the same size as rat CRABP on a Western blot of stage 24 chick limb buds (Maden *et al.* 1988).

Sections through the flank of stage 18 embryos in nonlimb regions show virtually no labelling of the mesodermal cells (Fig. 4). The epidermis is also unlabelled except for nonspecific reactivity on the surface. Sections through the region of the early limb bud, however, show intense labelling of the mesodermal cells, particularly those at the distal tip of the limb bud (Fig. 5). By stage 24, when the limb bud has elongated considerably and differentiation of the upper arm has begun proximally, several discrete regions of CRABP immunoreactivity can easily be distinguished (Fig. 6). At the tip, the apical ectodermal ridge is unlabelled (Fig. 7). The progress zone, which is the distalmost mesenchyme, is heavily immunoreactive. The cells in this location have a high rate of mitosis and are responsible for generating the complex pattern of elements that emerge sequentially during development (Wolpert *et al.* 1975). Most importantly, only such distal cells are able to respond to exogenously administered RA by duplicating their pattern (Tickle and Crawley, 1988). Behind the progress zone is undifferentiatied mesenchyme which does not label so intensely. In this region cells have begun to withdraw from the cell cycle in preparation for differentiation. Further proximally, differentiation can be seen to have commenced. In the central region, the humerus has begun to differentiate in the absence of CRABP immunoreactivity whereas the muscle and connective tissue on the periphery stain intensely.

The high levels of CRABP in the progress zone are likely to be of significance for the establishment of pattern in the limb bud. To examine this in further detail, we looked at the distribution of CRABP immunoreactivity across the AP axis, particularly with regard

Figs 4–8. Sections of chick embryos treated with an affinity-purified rabbit anti-rat CRABP antibody to reveal areas of specific immunoreactivity which are shown by the brown HRP reaction product. 4, section through the flank of a stage 18 embryo adjacent to the forelimb. There is no staining of the mesenchymal cells beneath the epidermis. Bar = 100 μm. 5, section through the limb bud of a stage 18 embryo. In contrast to Fig. 4, these mesenchymal cells are CRABP immunoreactive particularly at the distal tip. Bar = 100 μm. 6, stage 24 limb bud, longitudinal section, showing several areas of varying intensity of immunoreactivity. At the tip is the apical ectodermal ridge (aer) which does not label (see Fig. 7). The distalmost mesenchyme is the progress zone (pz) which is intensely CRABP reactive. Behind that is a weakly staining region consisting of undifferentiated mesenchyme (um). Further proximal the humerus (h) has differentiated in the absence of CRABP whereas the muscle, connective tissue and dermis (ct) stain intensely, particularly the dermis on the periphery. Bar = 150 μm. 7, high power view of the AER (arrow) showing the absence of staining in the epidermis compared to the mesenchyme of the progress zone beneath. Bar = 25 μm. 8, transverse section through the progress zone of a stage 21 limb bud. The anterior margin on the right is more intensely labelled than the posterior margin on the left. Bar = 100 μm.



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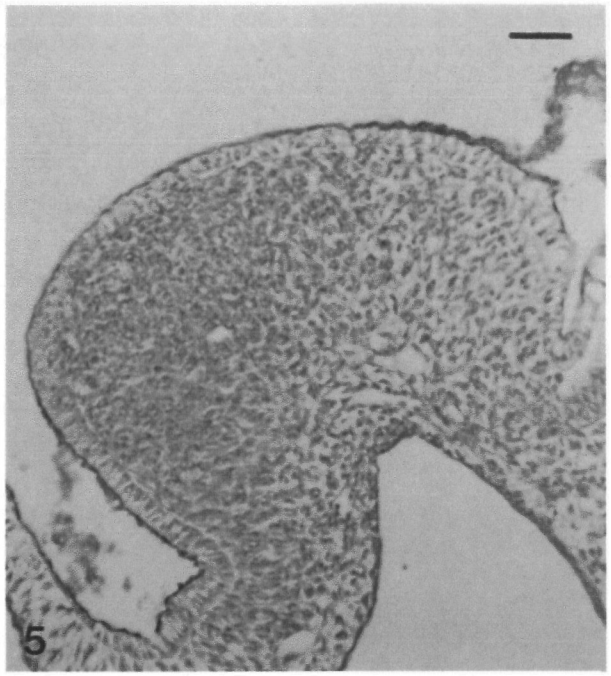

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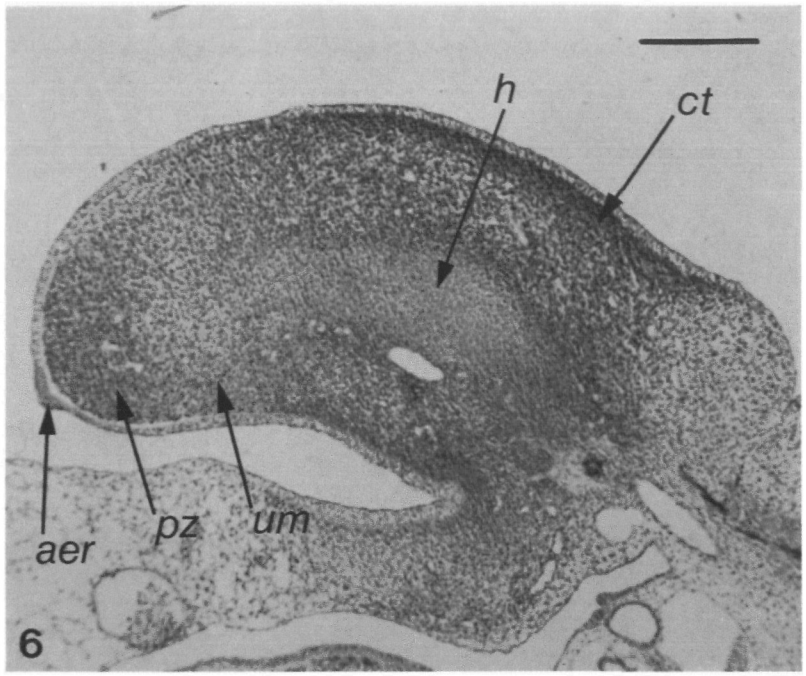

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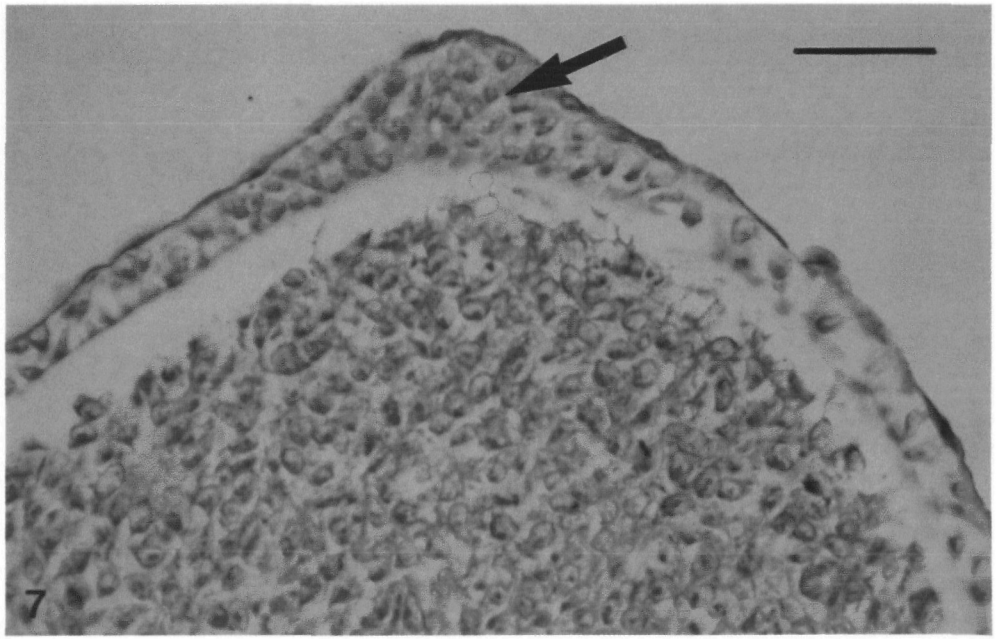

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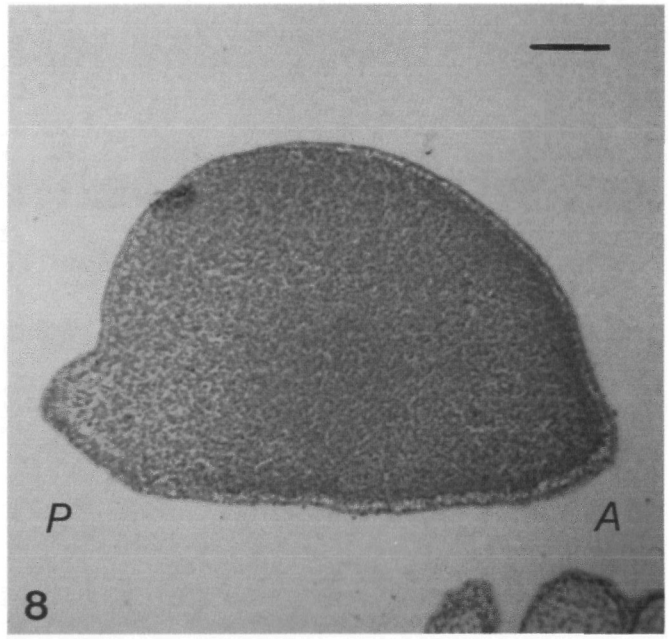

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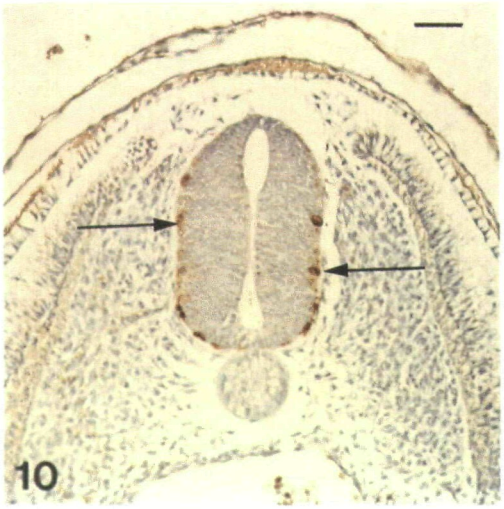
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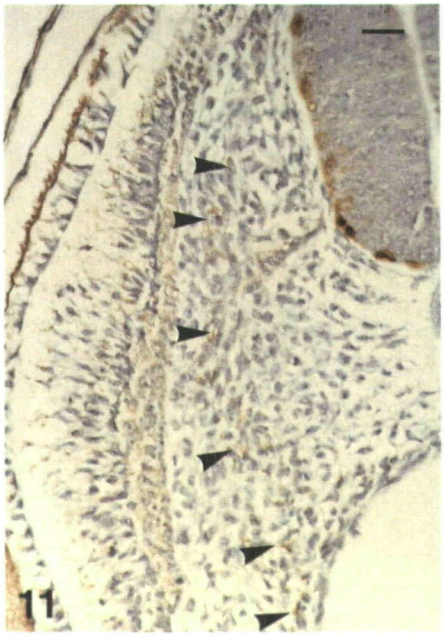
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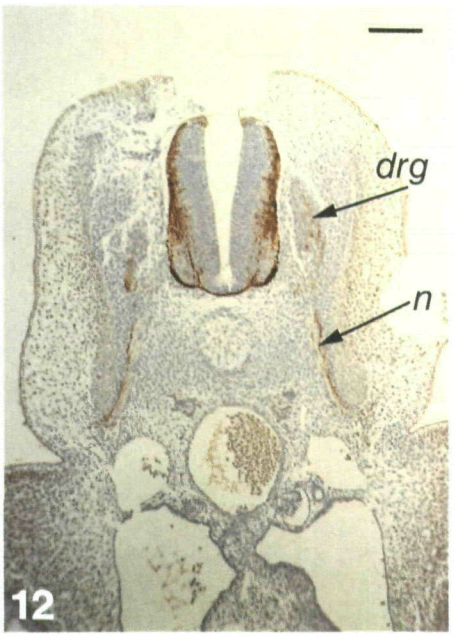

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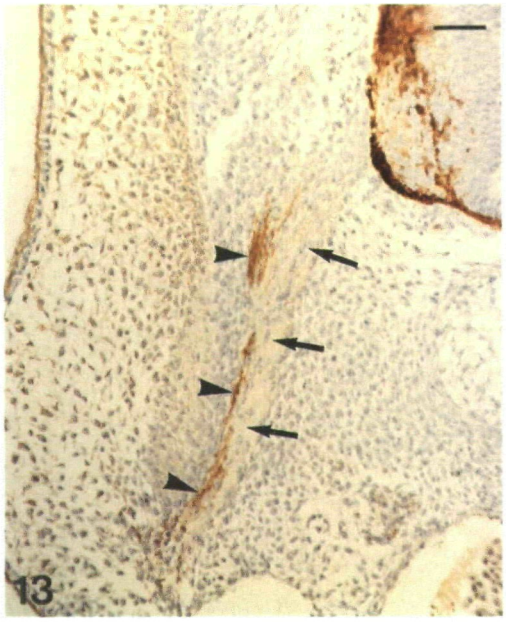
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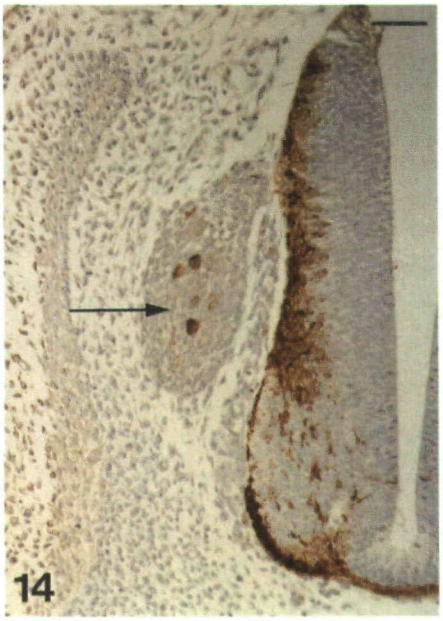
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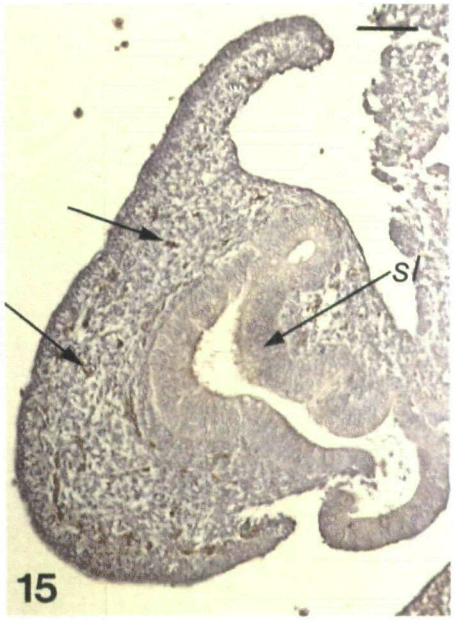

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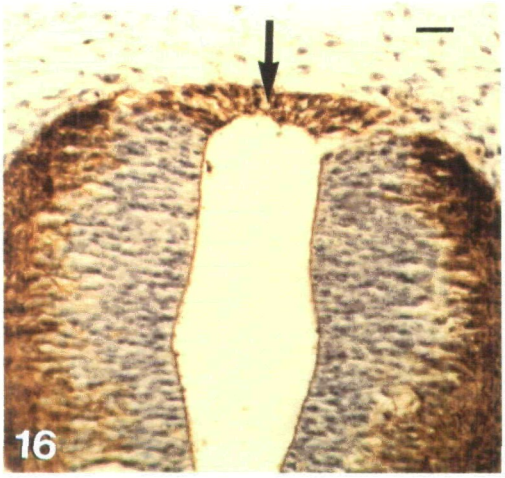
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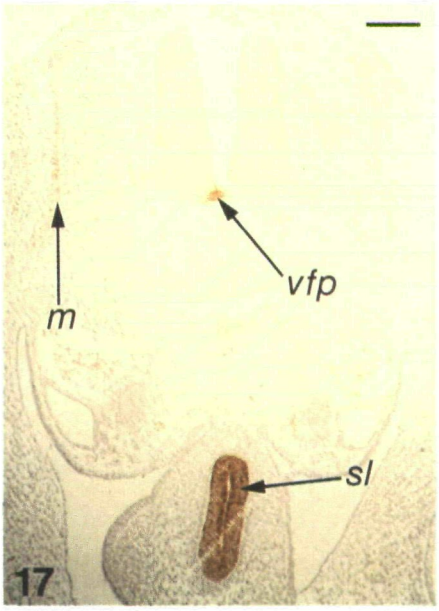

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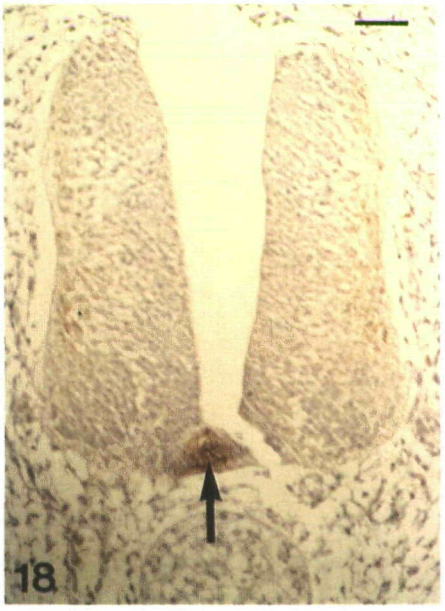
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to the endogenous gradient of RA which has its high point at the posterior side of the limb bud (Thaller and Eichele, 1987). In transverse sections, a gradient of CRABP was indeed detected, but to our surprise it was of the opposite polarity to that of RA, namely the high point was at the anterior margin and the low point at the posterior margin (Fig. 8). We have quantified the levels of CRABP using a false colour-image analysis system which gives a ratio of peroxidase label from anterior to posterior of 3.5:1.

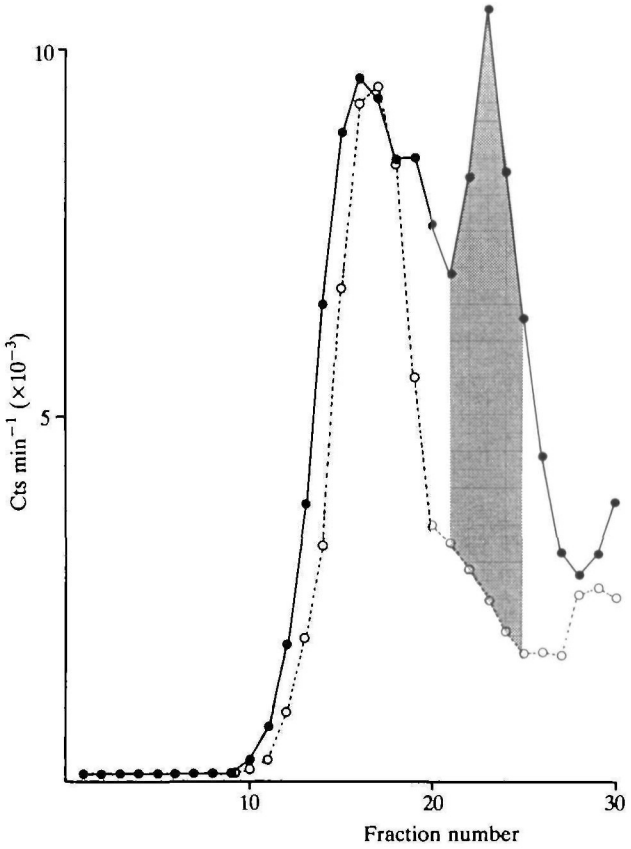

Fig. 9. Sucrose density gradient profile of stage 24 chick embryo cytosol to detect the presence of CRABP. 375 μg protein was incubated with 66 nM-[^{3}H]all-*trans*-retinoic acid (NEN, specific activity 55 Ci mmol^{-1}) alone (●——●) or in the presence of 100× M excess cold all-*trans*-retinoic acid (○---○). In these preparations, there are two peaks of radioactivity. The right-hand peak at approx. 17K which disappears in the presence of excess cold RA measures CRABP (hatched area). The left-hand peak at approx. 66K is non-specific.

It is important to note that these distributions of CRABP immunoreactivity are not caused by local variations in cell density. The two areas at the tip of the limb bud (progress zone and undifferentiated mesenchyme) have the same cell density, yet have a twofold variation in immunoreactivity and the anterior and posterior sides have similar densities despite a 3.5-fold difference in immunoreactivity.

Regarding the immunocytochemical distribution of CRBP, we cannot detect any in the limb bud. Coupled with the observation that only very low levels are present on sucrose gradient assays (Fig. 3) and that retinol is not an active compound when administered locally (Table 2), this suggests that CRBP may not be involved in the establishment of pattern in the chick limb bud.

Speculations

The levels of CRABP in a limb bud cell from birth to differentiation are thus strictly regulated. At birth in the progress zone CRABP levels are high in the rapidly dividing population of cells. As a cell leaves this zone, division slows down in preparation for differentiation and CRABP levels decline. As the developmental decision to become either cartilage or connective tissue/dermis is made CRABP levels are either extinguished (cartilage) or rise again (connective tissue/dermis). These fluctuations suggest that CRABP is playing a role in the generation of pattern in the limb bud.

In the proximodistal axis, CRABP may be responsible for maintaining a high rate of cell division in the progress zone. RA is known to stimulate proliferation in cultured distal mesodermal cells of the limb bud at concentrations that exist endogenously (Ide and Aono, 1988). So CRABP may mediate this function of RA by interacting with the retinoic acid receptors and ultimately the genes controlling proliferation.

Figs 10–18. Sections of chick embryos treated with affinity-purified rabbit anti-rat CRABP antibody (Figs 10–16) or rabbit anti-rat CRBP antibody (Figs 17–18) to reveal areas of specific immunoreactivity which are shown by the brown HRP reaction product. 10, section through the trunk at the forelimb level of a stage 16 embryo showing CRABP labelled cells in the mantle layer of the neural tube (arrows). Bar = 50 μm. 11, higher power view of Fig. 10 showing a string of cells (arrowheads) in the sclerotome which are CRABP immunoreactive. Bar = 25 μm. 12, section through the trunk at the forelimb level of a stage 22 embryo to reveal intense CRABP labelling on the periphery of the neural tube as well as in the nerves (n) and dorsal root ganglia (drg). Bar = 100 μm. 13, higher power view of Fig. 12 showing that only the sensory axons are immunoreactive (arrowheads) whereas the adjacent motor axons are unlabelled (arrows). Bar = 50 μm. 14, higher power view of Fig. 12, to show the labelling of individual cell bodies in the dorsal root ganglion (arrow). Bar = 50 μm. 15, section through the developing gut of a stage 22 embryo showing individual CRABP-positive cells in the thick gut wall (arrows). The secretory lining (sl) does not label in contrast to Fig. 17. Bar = 50 μm. 16, section through the neural tube of a stage 24 embryo which reveals intense CRABP reactivity in the dorsal roof plate (arrow). Bar = 25 μm. 17, CRBP immunoreactivity in a stage 24 embryo showing that only three areas label. One is the ventral floor plate (vfp), another is the secretory lining of the gut (sl) and the other is the myotome (m). Bar = 100 μm. 18, higher power view of Fig. 17 to show CRBP labelling in the cells of the ventral floor plate (arrow) rather than the fibres passing beneath which are CRABP positive (Figs 13 and 14). Bar = 50 μm.

In the anteroposterior axis, the role that CRABP might play seems different depending on whether we consider normal development or the results of experimental interference. In normal development, the gradients of RA and CRABP are of opposite polarity. This would result in a flat distribution of holo-CRABP thereby losing the positional differences that were generated in the first place. We have suggested (Maden *et al.* 1988) that the function of CRABP in the AP axis is to steepen the gradient of free RA. The 2.5-fold enrichment of endogenous RA in the posterior region would result in a change of only 15% in maximum binding across the limb which is rather small to specify the complexities of limb structure. A steeper gradient would increase this difference and may also lower the overall concentration of free RA (measured as 10^{-8}) so that it would be able to interact with the receptor (K_d 10^{-9}–10^{-10} M).

In the case of experimental interference, the gradient of CRABP with a high point at the anterior side conveniently explains why a ZPA graft or a RA implant only works to maximum effect when placed on the anterior side (Tickle *et al.* 1975, 1985). The concentration of holo-CRABP would be too low on the posterior side to cause a change in gene activity. When placed in the centre of the limb bud a ZPA graft or a RA implant is attenuated and this may be because the CRABP concentration is lower than at the anterior margin resulting in the transference of less RA to the nuclear receptors. In other situations this type of explanation is readily applicable. For example when fragments of the limb bud are cocultured, an anterior quarter with a posterior quarter results in far greater growth and cartilage differentiation than do two anterior quarters or two posterior quarters (Suzuki and Ide, 1987). The extra growth comes from the anterior fragment under the influence of the posterior one and these authors suggest that the lack of responsiveness of the posterior fragment itself may be due to the lack of a receptor. With a high CRABP concentration in the anterior fragments and a high RA concentration in the posterior fragments these results are readily explicable.

However, we are now left with two different mechanisms of action depending on whether we are considering the normal limb or the experimental limb, which is rather unsatisfying. Once again only with more information, particularly the involvement of the nuclear receptors, can we hope to unravel these complexities.

Finally, in the interests of universality it is worth mentioning that CRABP has been detected at high levels in the mouse limb bud (Kwarta *et al.* 1985). We have confirmed this result and the fact that CRBP is also present in mouse limb buds at about 1/10th the levels of CRABP (Table 1). So the same principles may apply throughout vertebrate limb development.

The developing spinal cord of the chick

CRABP

When the amount of CRABP in protein preparations from whole chick embryos is estimated by sucrose gradient analysis, high levels are observed (Fig. 9). In this preparation, as distinct from isolated limb buds (Fig. 1), nonspecific binding is seen at a higher molecular weight (approx. 66K) and is caused, at least in part, by [^{3}H]RA binding to albumin in the blood. The amount of CRABP in embryos is of the same order as that found in limb buds (Table 1).

Thus, with immunocytochemistry, we might expect to find extensive staining and that is indeed the case. Stage 16 to 24 embryos have been sectioned to look at the tissue distribution of CRABP. The myotome showed light staining at each stage and particularly at later stages the dermal and connective tissue cells showed immunoreactivity. But the most intensely stained cells were of neural or neural crest origin.

At stage 16 individual cells in the mantle layer of the neural tube particularly on the lateral and ventral sides had high levels of CRABP (Fig. 10). There appeared to be two populations of labelled cells, large cells that may be postmitotic early differentiating neurons and small, flattened cells particularly noticeable in the ventral floor plate region. These may form the pia on the outside of the neural tube. Certain neural crest cell populations were also heavily immunoreactive. There was a line of labelled cells stretching from the dorsal neural tube through the sclerotome to the lateral edge of the dorsal aorta (arrowheads in Fig. 11). These cells were present only in the anterior half of each sclerotome suggesting that they were neural crest cells in the process of migrating.

At stage 18, the mantle layer cells of the neural tube were again strongly immunoreactive as were individual cells between the neural tube and the myotome aggregating at the future site of the dorsal root ganglion. An additional site of localisation was a group of cells on each lateral edge of the dorsal aorta.

By stage 22, immunoreactivity in the neural tube had become much more extensive and intense (Figs 12–14). Cells of the mantle layer, dorsal roof plate and commissural axons which pass medial, lateral and through the motor horns and cross the ventral floor plate were labelled. Some of the dorsal root ganglion cells were labelled (Fig. 14) and particularly striking was immunoreactivity in axons passing out from the ganglion (arrowheads in Fig. 13). These axons maintained a dorsal position as they joined motor axons from the ventral horn and thus the two subpopulations of axons in the mixed nerve could clearly be seen. An additional site of immunoreactivity which first appeared at this stage was in individual cells of the walls of the developing gut (Fig. 15). It is likely that these are migrating neural crest cells which will form the enteric ganglia.

At stage 24, the distribution of immunoreactivity had not appreciably changed from stage 22. The dorsal roof plate labelling became more discrete (Fig. 16), a greater number of cells in the dorsal root ganglia were labelled and the selective staining of sensory axons in the brachial plexus was more apparent.

CRBP

Sucrose gradient assays revealed that the embryo had

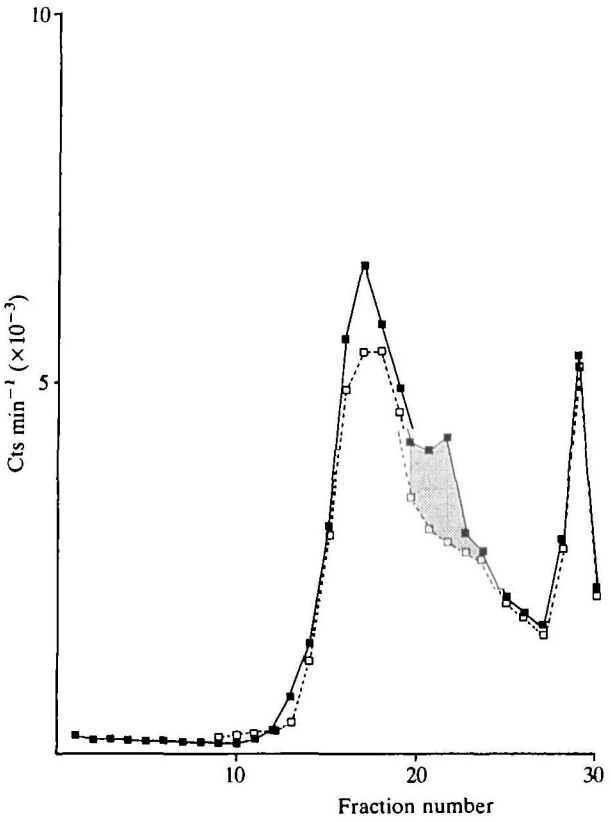

Fig. 19. Sucrose density gradient profile of stage 24 chick embryo cytosol to detect the presence of CRBP. μg protein was incubated with 66 nM-[^{3}H]all-*trans*-retinol (Amersham, specific activity 60 Ci mmol^{-1}) alone (■——■) or in the presence of 500× M excess cold all-*trans*-retinol (□---□). As in Fig. 9 there are two peaks of radioactivity, one is non-specific (left) and one is specific for CRBP (right). Although equivalent amounts of protein were used, the level of CRBP (hatched area) is far less than the level of CRABP measured in Fig. 9.

low levels of CRBP (Fig. 19, Table 1). Like the limb bud there was about 1/10th of the amount of CRBP in the embryo as CRABP.

We did not therefore expect to see very much in our immunocytochemical analysis, which proved to be the case. However, the distribution of immunoreactivity was very surprising. At all stages examined, the secretory lining of the developing gut showed intense labelling (Fig. 17). This is in obvious contrast to CRABP immunoreactivity, which highlighted individual cells in the walls not the epithelium (Fig. 15).

The second area of immunoreactivity was the ventral floor plate cells (Fig. 18). Again, in contrast to CRABP where the commissural fibres passing *below* the floor plate labelled (see Figs 13 and 14), with CRBP it was the cells of the floor plate itself that were labelled. Also, in contrast was the labelling of the dorsal roof plate with CRABP (Fig. 16) and the ventral floor plate with CRBP.

The only other area of immunoreactivity was the myotome (Fig. 17).

Speculations

The patterns of CRABP and CRBP immunoreactivity in the trunk of the chick embryo show discrete and striking contrasts in three areas: the neural tube, the neurons and the gut.

In the neural tube, CRABP reactivity is found in cells in the mantle layer which become the commissural neurons and in the dorsal roof plate. CRBP is found only in the ventral floor plate. The ventral floor plate is an important decision-making region which first attracts commissural axons towards it and then causes these axons to make abrupt right-angled turns to project in the rostrocaudal axis along the lateral surface of the floor plate (Dodd and Jessell, 1988; Tessier-Lavigne *et al.* 1988). In the light of the observations reported here, it is possible that the ventral floor plate is rich in retinol, which it metabolises to RA and releases as a chemoattractant. Since commissural fibres have CRABP only, they are able to take up RA and respond accordingly by growing towards the source. This attractive theory can initially be tested by determining whether RA or perhaps retinol are indeed chemoattractants for neurons and specifically commissural neurons.

Sensory neurons are highly immunoreactive to CRABP whereas motor neurons are not. This suggests that RA may play a role in the decision-making process involved in the differentiation of sensory *vs* motor nerves. Alternatively RA may be involved in the guidance of axons into, for example, the limb in the same way that was proposed above for commissural axons. Again it would be a simple matter to test whether RA is a chemoattractant for nerves.

The secretory lining of the gut labels with CRBP whereas individual cells in the thick wall of the gut label with CRABP. These CRABP-positive cells are most likely to be neural crest cells migrating through the gut walls to form the enteric plexus (Maden *et al.* 1989). CRBP is present in the epithelium of the rat intestine (Ong *et al.* 1982) and we would therefore expect to find it in the chick embryo. However, in rats there is another distinct species of CRBP, CRBP(II) (Ong, 1984), which is present in the intestinal epithelium at 1000× the level of CRBP (Crow and Ong, 1985). It is possible that the immunoreactivity we have described here is detecting chick CRBP(II) which in the chick may be less immunologically distinct from CRBP than in the rat. In any case, the location of this CRBP immunoreactivity implies a role in the absorption of retinol from the gut.

Concluding remarks

The above data on the levels and localisation of CRABP and CRBP in the regenerating amphibian limb, the chick limb bud and the chick nervous system provide evidence to support the view that these binding proteins and their ligands, retinol and retinoic acid, are involved in the generation of pattern in these systems.

Interestingly, similar localisations have recently been reported, particularly in the limb, concerning homoeobox genes. In *Xenopus* and mouse the XLHbox 1

protein is localised in the forelimb bud in the form of a gradient with the high point on the anterior side (Oliver *et al.* 1988). It is also strongest in proximal regions of the limb and absent at the distal tip and absent from hindlimb buds. The Hox-7 transcript is present in the mouse initially throughout the limb bud, but later it localises to the distal and posterior periphery under the ectoderm and later still to interdigital mesenchyme (Robert *et al.* 1989; Hill *et al.* 1989). In the chick, Ghox 2.1 has been investigated with *in situ* hybridisation to reveal a patch of expression at the anterior margin of the chick limb bud in a proximal region (Wedden *et al.* 1989).

In addition, these genes are also expressed in the nervous system as are many other homoeobox genes (Holland and Hogan, 1988). Thus there is an association both in the limb and the nervous system between homoeobox genes, CRABP/CRBP and retinoids. Since RA is known to be able to activate the transcription of homoeobox genes (Mavilio *et al.* 1988) these three components may be intimately associated with the generation of pattern in the embryo.

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