J. Embryol. exp. Morph. **97**, 239–250 (1986)
Printed in Great Britain © *The Company of Biologists Limited 1986*

Retinoic acid-binding protein in the chick limb bud: identification at developmental stages and binding affinities of various retinoids

M. MADEN AND D. SUMMERBELL
Laboratory of Neurobiology and Development, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

SUMMARY

The application of retinoic acid (RA) to the developing chick limb bud causes 6-digit double posterior limbs to form instead of the normal 3-digit limb. As an attempt to begin a molecular analysis of this phenomenon we have identified and characterized a soluble cytoplasmic receptor for RA, namely cytoplasmic retinoic acid-binding protein (CRABP), from the cells of the chick limb bud. It is present from stages 20–35 at similar levels and has an apparent K_d of 140–280 nM. In competition experiments with other retinoids Ro 13-7410 was found to be the most effective at competing for sites on CRABP followed by all-*trans*-RA, 13-*cis*-RA, Ro 10-1670 and retinal. Retinol, retinyl palmitate, retinyl acetate, etretinate and arotinoid showed low or no affinity for CRABP. Specificity for binding was thus demonstrated since analogues with an acid end group competed effectively, the aldehyde competed less effectively and the ester or alcohol groups did not compete. At the concentration of RA that needs to be administered to cause duplications in the pattern of the limb bud, we estimate that 4% of the CRABP present in the limb bud has RA bound. The similarities between steroid receptors in the mediation of steroid hormone action and CRABP in the mediation of RA action is discussed. In this regard we note that while there are 10^4 steroid receptors per cell in other cell types we estimate that there are about 10^5 RA receptors per cell in the chick limb bud.

INTRODUCTION

Vitamin A and its analogues, the retinoids, have long been a subject of research in the fields of vision, nutrition, metabolism and differentiation. Most recently they have aroused great interest because of their unique effects on pattern formation in developing and regenerating limbs. These compounds have the ability to change the positional information of cells in the limb in a very precise manner.

When retinoic acid (RA) is applied locally to the anterior side of the developing chick limb bud it causes mirror-image duplications in the anteroposterior axis such that a 6-digit double posterior limb develops instead of the normal 3-digit one (Tickle, Alberts, Wolpert & Lee, 1982; Summerbell, 1983). In developing amphibian limbs, retinyl palmitate causes duplications in both the anteroposterior axis and the proximodistal axis (Niazi & Saxena, 1978; Maden, 1983), the net result of

Key words: limb bud, retinoic acid, retinoic acid-binding protein, pattern formation, chick embryo, retinoids, binding affinities, steroid receptors.

which can be the production of a complete pair of limbs including the girdle growing out from the foot. The regenerating limbs of amphibians also duplicate in the proximodistal axis after retinoid administration (Maden, 1982; Thoms & Stocum, 1984) and can be made to reveal anteroposterior effects after appropriate surgery (Stocum & Thoms, 1984).

As far as mechanisms are concerned we have no idea of precisely what retinoids are doing to limb cells when they are respecified in this manner. However, in the chick limb bud, the striking similarity between retinoic acid effects and the result of grafting the zone of polarizing activity (Tickle, Summerbell & Wolpert, 1975) led to the suggestion that either retinoic acid itself is a morphogen or that it is acting locally to convert adjacent tissue into a signalling region (Tickle, 1983; Summerbell, 1983). The similarities extend not only to the obvious morphological structure, but also to dose dependency, position effects and time dependency. Most recently, data showing that retinoic acid becomes distributed across the anteroposterior axis of the chick limb bud in the same graded fashion as the putative morphogen released by the zone of polarizing activity have provided support for the former suggestion (Tickle, Lee & Eichele, 1985; Eichele, Tickle & Alberts, 1985). More than this we do not yet know, but some insights may be gained by looking at how retinoids are thought to act in other systems.

One view of the action of retinoids on cells is the steroid model whereby retinoids enter the cell, bind to a specific cytoplasmic receptor, are transported to the nucleus and change the pattern of gene transcription, which results in altered cell morphology (Roberts & Sporn, 1984). The involvement of specific cytoplasmic receptors, the retinoid-binding proteins, is central and considerable evidence has accumulated in support of this contention. For example, the distribution of cytoplasmic retinol-binding protein (CRBP) and cytoplasmic retinoic acid-binding protein (CRABP) in various tissues, mostly of mammalian origin, correlates with the requirement of that tissue for vitamin A (Chytil & Ong, 1984). CRABP is usually present at high levels in tumours, but at low levels or absent in the corresponding normal tissues (Ong, Page & Chytil, 1975; Palan & Romney, 1980; Mehta, Cerny & Moon, 1980; Ong, Goodwin, Jeese & Griffin, 1982). Teratocarcinoma cells and other cancer cell lines which normally respond to retinoic acid contain CRABP, but mutants selected for their lack of responsiveness have lost their binding proteins (Schindler, Matthaei & Sherman, 1981; Wang & Gudas, 1984; Marth, Mayer & Daxenbichler, 1984).

Thus an obvious question to ask with regard to the action of retinoic acid (RA) on the chick limb bud is whether it contains CRABP at stages that are susceptible to RA-induced pattern alterations. We report here the detection and partial characterization of CRABP from the cytoplasm of the cells of the chick limb bud, the binding of various analogues to this receptor and discuss its possible role in RA-induced pattern respecification.

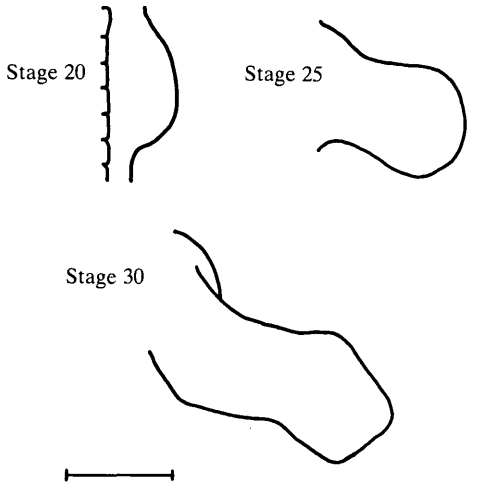


Fig. 1. *Camera lucida* drawings of chick limb buds to show the stage of development at which the experiments were performed. Bar, 1 mm.

MATERIALS AND METHODS

Fertilized chick eggs from a mixed local flock (Needle Farm) were incubated to stages 20–35 (Fig. 1). At stages 20, 21, 22, 25, 30 and 35 forelimbs were removed from embryos and frozen in liquid nitrogen until enough material had been accumulated.

Retinoids

[11,12-^{3}H]all-*trans*-retinoic acid (30 Ci mmol^{-1}) and the synthetic analogues Ro 10-9359 (etretinate: ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate), Ro 10-1670 (all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid), Ro 13-7410 (*p*-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl] benzoic acid) and Ro 13-6298 (arotinoid: ethyl *p*-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl] benzoate) were kindly provided by Hoffman-La Roche & Co. Ltd, Basel. 13-*cis*-retinoic acid was a gift from Dr C. Tickle, The Middlesex Hospital Medical School, London, and all other retinoids were purchased from Sigma. The purity of all-*trans*-RA and 13-*cis*-RA was determined by reverse phase HPLC using a μBondapak C_{18} column with 0·01 M sodium acetate methanol: water 80:20 as the buffer (McCormick, Napoli & DeLuca, 1978).

Cytosol preparation

Limb buds were homogenized in 1 ml of ice-cold phosphate-buffered saline (PBS = 0·03 M sodium phosphate buffer, pH 7·2 in 0·1 M NaCl) in a Dounce homogenizer. Samples were then spun at 100000 *g* for 1 h at 4 °C in a Spinco SW50.1 rotor. The protein content of the supernatant was measured with the Pierce protein assay reagent (Pierce Chemical Company, Rockford, Illinois) using bovine serum albumin as the standard. The preparations were then aliquotted and stored at −80 °C until use.

Binding assay

Samples for assays contained about 400 μg of protein in 40–125 μl. Each was diluted to 500 μl with PBS, 66 nM of [^{3}H]all-*trans*-retinoic acid was added in 10 μl dimethylsulphoxide (DMSO) with or without 100 × molar excess of unlabelled all-*trans*-RA, also in 10 μl DMSO. The mixtures were incubated at 4 °C for 4 h. They were then dialysed against PBS for 24 h at 4 °C, placed on 4·5 ml 5–20 % sucrose gradients and spun at 180000 *g* for 17 h in an SW50.1 rotor at

4 °C using a Beckman L2-65B Ultracentrifuge. Gradients were separated into five drop fractions (180 μl), the refractive index of each fraction was checked and radioactivity counted in 10 ml Liquiscint (National Diagnostics).

Bovine serum albumin (BSA) and myoglobin were used as relative molecular mass markers (4·6 S and 2 S) on separate gradients, their positions on the gradient being determined by the OD_{280} reading. Specific binding was demonstrated with the addition of 100 × molar excess cold all-*trans*-RA and quantified by determining the drop in cts min^{-1} in the 2 S region of the gradient (between fractions with refractive index 1·3425 and 1·3475). The values obtained assumed a single binding site for retinoic acid and were for comparisons between stages and not intended to be absolute measures of the amount of CRABP in limb buds.

Saturation analysis

Protein samples (400 μg in 500 μl PBS) were incubated at 4 °C for 4 h with increasing concentrations of ^{3}H-RA from 1–220 nM with or without 100 × molar excess of unlabelled all-*trans*-RA. They were dialysed, spun and fractionated as described above. Specific binding was calculated and a Scatchard analysis performed.

Competition studies

For competition studies, protein samples (400 μg protein in 500 μl PBS) were incubated for 4 h at 4 °C with 130 nM ^{3}H-RA in 10 μl DMSO and from 0·33 to 500 × molar excesses of unlabelled analogues also in 10 μl DMSO. The analogues added were retinol, retinyl palmitate, retinyl acetate, retinal, 13-*cis*-RA, Ro 10-9359, Ro 10-1670, Ro 13-7410 and Ro 13-6298. Samples were then dialysed for 24 h, spun on a sucrose gradient and fractionated as described above. The decrease in binding caused by the analogues was quantified and plotted against concentration of ligand. The 50% intercept (IC_{50}) was used as a comparative value.

RESULTS

Presence of CRABP at various stages

We began by examining stage 20 limb buds (Fig. 1), which are still susceptible to pattern changes induced by retinoic acid (Summerbell & Harvey, 1983). At this stage the limb bud forms a small protrusion from the flank of the embryo, consisting of an apparently homogeneous mass of mesenchymal cells surrounded by a thin layer of ectoderm. Cytoplasmic protein preparations incubated with 66 nM [^{3}H]*trans*-retinoic acid (^{3}H-RA) showed a single peak of radioactivity on a 5–20% sucrose gradient (Fig. 2A). The peak cosedimented with myoglobin run on a separate gradient, suggesting an approximate relative molecular mass of 17000, and could be abolished when incubated in the presence of 100 × molar excess cold *trans*-RA (Fig. 2A). This indicated the presence of a specific, saturable receptor for retinoic acid.

At later stages of limb development, differentiation begins and the characteristic shape of the limb is manifest (Fig. 1). These stages also have CRABP present (Fig. 2B–D). Indeed, at all stages tested (20, 21, 22, 25, 30, 35) the receptor was present at approximately similar levels per mg of cytosolic protein (Table 1), although there is some suggestion of a decrease at stages 25 and 30. These levels are similar to those found in embryonic chick skin (Maden & Summerbell, unpublished data), a tissue that is also susceptible to retinoid-induced pattern changes (Dhouailly, 1982). Thus CRABP is present during the

early stages of limb development that are sensitive to the patterning effect of RA (around stage 20) and also at later stages when teratogenic effects generally appear after RA administration (Summerbell, 1983).

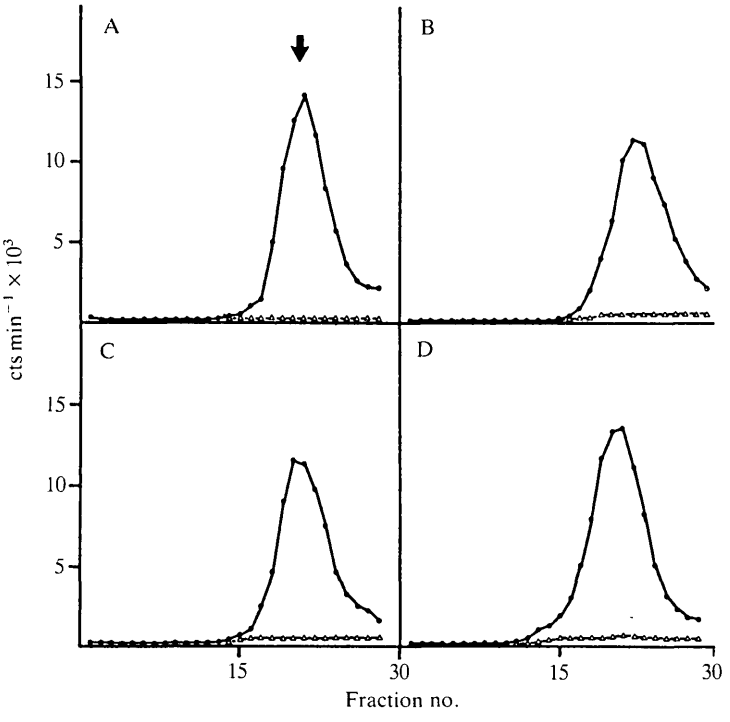


Fig. 2. Sucrose density gradient profiles of chick limb bud cytosols from various stages after incubation with 66 nM [^{3}H]all-*trans*-retinoic acid alone (●——●) or in the presence of 100 × M excess cold all-*trans*-RA (△---△). The single peak of radioactivity which disappears with excess cold all-*trans*-RA indicates the presence of a specific, saturable receptor for retinoic acid, cellular retinoic acid-binding protein (CRABP). The peak of radioactivity coincides with the position of myoglobin (arrow) run on a separate gradient suggesting a relative molecular mass in the range of 17 000. (A) Stage 20; (B) stage 25; (C) stage 30; (D) stage 35.

Table 1. *Estimates of the amount of CRABP in the chick limb bud at various stages of development*

Stage	Amount (pmoles mg^{-1} cytosolic protein)
20 (n = 5)	6·08 ± 0·52*
25 (n = 5)	4·76 ± 0·22
30 (n = 3)	4·46 ± 0·2
35 (n = 5)	6·66 ± 0·8

* ±S.D.
See Fig. 1 for drawings of the stages.

Saturation analysis

Scatchard plots of several sets of data from stage 25 and 30 CRABP gave estimates of 140–280 nM for the dissociation constant (K_d), which is at the upper end of the range of K_d values from other tissues. These estimates vary from 2 nM (mouse limb buds: Kwarta, Kimmel, Kimmel & Slikker, 1985) and 4·2 nM (rat testis: Ong & Chytil, 1978) to 318 nM (rat testis: Bonelli & DeLuca, 1985) and 400 nM (human skin: Siegenthaler & Saurat, 1985). The total specific binding capacity was estimated to be 14–28 pmoles CRABP mg^{-1} cytosol protein, the same value obtained from mouse limb buds (Kwarta *et al.* 1985). The plots were consistent with a single binding site.

Competition studies

The relative binding affinities of various vitamin A analogues to this chick limb bud CRABP were determined in competition experiments. We first examined the competition with 100 × molar excesses of each of nine analogues. Retinol, retinyl palmitate, retinyl acetate, etretinate (Ro 10-9359) and arotinoid (Ro 13-6298) did not compete for binding to CRABP (Fig. 3A; Table 2). Increasing the molar excess of arotinoid to ×300 or etretinate to ×500 did not reveal any competition either. The terminal end group of etretinate and arotinoid is $-COOC_2H_5$: thus neither esters nor the alcohol can compete for binding to CRABP.

On the other hand, retinal did compete, but not as effectively as all-*trans*-RA, whereas 13-*cis*-RA, Ro 13-7410 and Ro 10-1670 did compete as effectively as all-*trans*-RA at 100 × molar excess (Fig. 3A).Ro 10-1670 is the free acid of etretinate and Ro 13-7410 is the free acid of arotinoid. Thus the binding to CRABP is largely specific for an acid end group although the aldehyde can compete to a certain extent.

We then examined in more detail the relative efficacies of the binding of retinal, 13-*cis*-RA, Ro 13-7410 and Ro 10-1670. We began by determining the 50% displacement level (IC_{50}) of all-*trans*-RA. Low excesses (×0·33 or 1) decreased significantly the binding of label and the IC_{50} was reached at a value of about ×3·5. When the displacement curve was plotted the molarity of all-*trans*-RA at IC_{50} was $1{\cdot}9\times10^{-7}$ M (Fig. 3B; Table 2). The same experiment using Ro 13-7410 gave a slightly lower value of $1{\cdot}0\times10^{-7}$ M, suggesting that this analogue may be more effective at binding to CRABP than its natural ligand. 13-*cis*-RA, the isomer of all-trans-RA, gave an IC_{50} of $6{\cdot}6\times10^{-7}$ M (Fig. 3B). This means that *cis*-RA is three to four times less effective at competing for sites on CRABP than all-*trans*-RA. Ro 10-1670 gave a value of $1{\cdot}8\times10^{-6}$ M, which is about ten times less effective than all-*trans*-RA, and retinal gave a value of $2{\cdot}2\times10^{-6}$ M, which is about twelve times less effective (Table 2).

We can therefore rank these analogues in order of binding efficacy to CRABP thus: Ro 13-7410 > all-*trans*-RA > 13-*cis*-RA > Ro 10-1670 > retinal and those with low or no affinity are retinol, retinyl palmitate, retinyl acetate, etretinate and arotinoid.

DISCUSSION

The graded response of the chick limb bud to RA, whereby more duplicated digits are produced as the dose increases suggested to Tickle *et al.* (1985) that a specific receptor protein may mediate the acquisition of positional information. Here we have demonstrated that there is indeed a specific cellular receptor for retinoic acid in the developing chick limb bud. It is present at least from stage 20 to stage 35, has an apparent K_d of 140–280 nM and shows specificity for an acid terminal group. But does this mean that CRABP plays a role in the mechanism of RA-induced pattern alterations? Several points can be made to support this contention.

First, we can estimate how the concentration of CRABP in the limb bud is related to the amount of RA needed to cause duplications. If RA is in an excess then it would be unlikely to act *via* CRABP because the receptor–ligand complexes would be saturated. Conversely, if the CRABP concentration is in excess compared to RA then it would be unlikely to be acting primarily as a receptor for RA. Tickle *et al.* (1985) have deduced that 7·6 pg of RA per limb bud gives a full duplication, which is 25 fmoles of RA. We estimate above that there is about 5 pmoles of CRABP per mg cytosol protein, which is 125 fmoles CRABP per limb bud. Therefore the amount of RA needed to cause duplications lies on the linear part of the binding curve where the percentage of sites occupied is proportional to the concentration of RA.

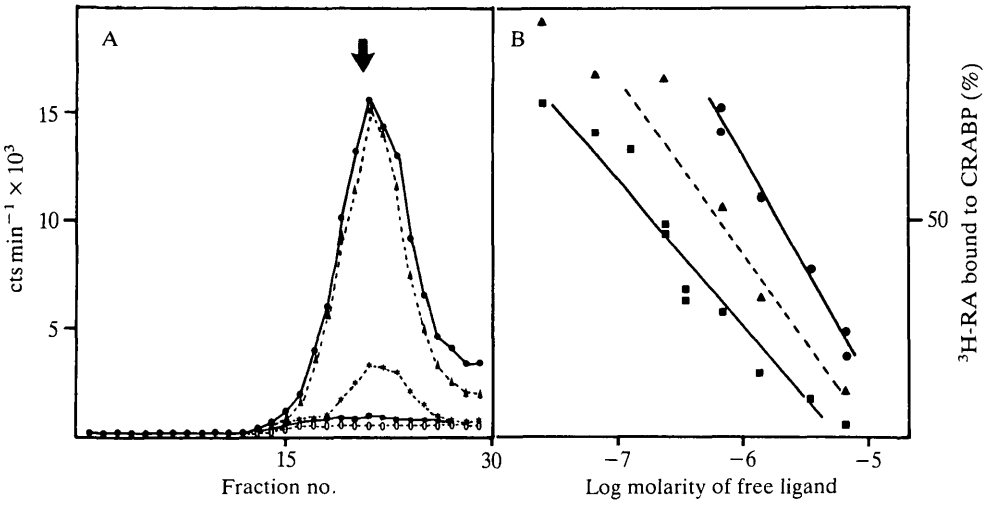
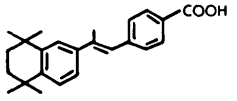
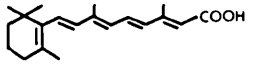
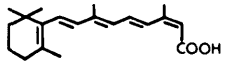
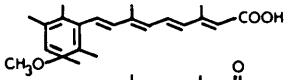
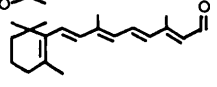
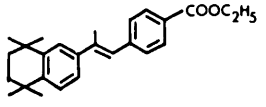
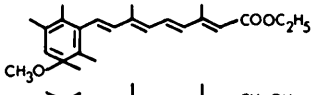
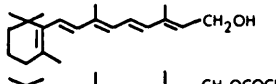
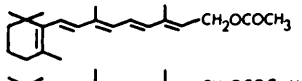
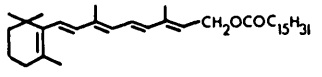


Fig. 3. (A) Competition with other analogues. Aliquots of stage 30 chick limb bud cytosols incubated with [^{3}H]all-*trans*-RA alone (●——●) or in the presence of 100 × M excess of retinyl palmitate (▲---▲), retinal (*---*), 13-*cis*-RA (■——■) or all-*trans*-RA (O---O). Retinol palmitate does not compete for binding of the labelled ligand to CRABP whereas retinal and 13-*cis*-RA do, the latter being more effective than the former. The arrow indicates the position of the myoglobin marker. (B) The displacement of bound [^{3}H]all-*trans*-RA from stage 30 CRABP by increasing molar excesses of all-*trans*-RA (■——■), 13-*cis*-RA (▲---▲) and Ro 10-1670 (●——●). The molarity of excess analogue (ordinate) at the 50% displacement level (abscissa) is the IC_{50} value recorded in Table 2.

Second, CRABP was present at high levels during those stages of limb development when RA changes the positional information of cells in the anteroposterior axis (around stage 20: Summerbell & Harvey, 1983). It is also present at similar levels in embryonic chick skin (Sani & Hill, 1976; Maden & Summerbell, unpublished data) and in this system RA changes the pattern of development by causing feathers to form instead of scales (Dhouailly, 1982). In the regenerating amphibian limb RA changes the positional information of blastemal cells (see Introduction) and the levels of CRABP are four times higher at RA-susceptible stages (Keeble & Maden, 1986*a*). These correlations suggest a role for CRABP in the mechanism of action of RA. Of course, it must be said that the mere presence of CRABP does not imply that pattern changes will, of necessity, occur. In the chick limb bud the capacity to regulate in the anteroposterior axis has almost disappeared by stage 24/25 (Summerbell, 1974) so despite the presence of CRABP at stages 25+, RA could not be expected to cause pattern alterations. CRABP has been identified in the mouse limb bud (Kwarta *et al.* 1985), but RA

Table 2. *Structures and binding efficacy of various retinoids to stage 30 chick limb bud CRABP*

Analogue	Structure	Competition at 100 × M excess	IC_{50}
Ro 13-7410 (TTNPB)		+	$1{\cdot}0\times10^{-7}$ M
all-*trans*-RA		+	$1{\cdot}9\times10^{-7}$ M
13-*cis*-RA		+	$6{\cdot}6\times10^{-7}$ M
Ro 10-1670		+	$1{\cdot}8\times10^{-6}$ M
Retinal		+	$2{\cdot}2\times10^{-6}$ M
Ro 13-6298 (arotinoid)		−	—
Ro 10-9359 (etretinate)		−	—
Retinol		−	—
Retinyl acetate		−	—
Retinyl palmitate		−	—

The IC_{50} value was obtained from the data in Fig. 3B and additional data not shown.

administration to pregnant mice only produces teratogenic effects on these embryonic limbs (Kochhar, 1977). Similarly in culture systems some cell lines are refractory to the effects of RA despite having normal levels of CRABP (Matthaei, Andrews & Bronson, 1983; Lacroix, L'Heureux & Bhat, 1984). Nevertheless, the evidence presented in the Introduction suggests that the presence of CRABP may well be a precondition for the action of RA.

Third, in both the chick limb bud (Maden & Summerbell, unpublished data) and the regenerating amphibian limb (Keeble & Maden, 1986*b*) we have shown that ^{3}H-RA enters the nuclei of limb cells. Thus the suggested mode of action of RA, whereby it enters the cells, binds to CRABP, the CRABP–RA complex is transported to the nucleus and changes gene transcription, may well be operating to change the positional information of limb cells. Indeed, changes in protein synthetic profiles in RA-treated regenerating blastemas have been identified on 2-D gels (Slack, 1983). We are currently exploring in greater detail this postulated mode of action.

Fourth, competition studies with retinoid analogues are performed to correlate binding activity with biological activity (Lotan, Neumann & Lotan, 1980; Trown *et al.* 1980; Sani *et al.* 1984). Such correlations, if they exist, provide evidence that CRABP mediates the biological effects of RA. Thus with chick limb bud CRABP we obtained the following binding efficacy: Ro 13-7410 > all-*trans*-RA > 13-*cis*-RA > Ro 10-1670 > retinal. Retinol, retinyl palmitate, retinyl acetate, etretinate and arotinoid showed little or no affinity (Table 2). So far we only know that Ro 13-7410 is more effective than all-*trans*-RA (Eichele *et al.* 1985), that all-*trans*-RA is more effective than 13-*cis*-RA (Tickle *et al.* 1985) and that retinyl palmitate does not appear to be active in the chick limb bud (Summerbell & Harvey, 1983). Ro 13-7410 is also the most active analogue in altering feather morphogenesis in chick skin (Cadi, Pautou & Dhouailly, 1984) and 13-*cis*-RA is less active than all-*trans*-RA as a teratogen of the mouse limb bud (Kochhar, Penner & Tellone, 1984). These limited biological efficacy data that we have fit well with the binding studies reported here although they ignore the possibility of differential uptake and differential metabolism of these retinoids by the cells of the limb bud (e.g. Eichele *et al.* 1985). They may be converted to active or inactive metabolites before binding to CRABP and thus invalidate the above comparisons. For example, etretinate can alter pattern formation in the regenerating amphibian limb, although it is less active than all-*trans*-RA (Kim & Stocum, unpublished data) but etretinate shows little or no affinity for amphibian blastemal CRABP (Keeble, unpublished data). However, etretinate is known to be metabolized to its free acid Ro 10-1610 (Paravicini *et al.* 1984) and this compound does bind to CRABP. With these provisos in mind the analogues used here for binding studies are currently being tested on the chick limb bud for their biological efficacy in inducing pattern alterations.

In conclusion, the above considerations provide evidence to support the steroid model as a possible explanation for the mode of action of retinoic acid on the chick limb bud. If this is the case and RA acts *via* the genome then we may be able to

identify the genes responsible for the control of pattern formation during limb development.

We thank Hoffmann-La Roche, Basel, for many generous gifts of labelled and unlabelled retinoids and Katriye Mustafa for excellent technical assistance.

REFERENCES

BONELLI, F. C. & DELUCA, L. M. (1985). A high-performance liquid chromatography technique that separates cellular retinol-binding protein from cellular retinoic acid-binding protein. *Analyt. Biochem.* **147**, 251–257.

CADI, R., PAUTOU, M.-P. & DHOUAILLY, D. (1984). Structure-activity relationships of retinoids in the morphogenesis of cutaneous appendages in the chick embryo. *J. invest. Dermat.* **83**, 105–109.

CHYTIL, F. & ONG, D. (1984). Cellular retinoid-binding proteins. In *The Retinoids*, vol. **2** (ed. M. B. Sporn, A. B. Roberts & D. S. Goodman), pp. 90–123. Orlando, Florida: Academic Press.

DHOUAILLY, D. (1982). Effects of retinoic acid on developmental properties of the foot integument in avian embryo. In *Embryonic Development, Part B: Cellular Aspects*, pp. 309–316. New York: Alan R. Liss.

EICHELE, G., TICKLE, C. & ALBERTS, B. M. (1985). Studies on the mechanism of retinoid-induced pattern duplications in the early chick limb bud: temporal and spatial aspects. *J. Cell Biol.* **101**, 1913–1920.

KEEBLE, S. & MADEN, M. (1986*a*). Retinoic acid-binding protein in the axolotl: distribution in mature tissues and time of appearance during limb regeneration. *Devl Biol.* (in press).

KEEBLE, S. & MADEN, M. (1986*b*). The presence of retinoic acid-binding proteins in amphibian tissues and their possible role in limb regeneration. In *New Discoveries and Technologies in Development.* New York: Alan R. Liss (in press).

KOCHHAR, D. M. (1977). Cellular basis of congenital limb deformity induced in mice by vitamin A. In *Morphogenesis and Malformations of the Limb* (ed. D. Bergsma & W. Lenz), pp. 111–154. New York: Alan R. Liss.

KOCHHAR, D. M., PENNER, J. D. & TELLONE, C. I. (1984). Comparative teratogenic activities of two retinoids: effects on palate and limb development. *Teratogenesis, Carcinogenesis and Mutagenesis* **4**, 377–387.

KWARTA, R. F., KIMMEL, C. A., KIMMEL, G. L. & SLIKKER, W. (1985). Identification of the cellular retinoic acid-binding protein (CRABP) within the embryonic mouse (CD-1) limb bud. *Teratology* **32**, 103–111.

LACROIX, A., L'HEUREUX, N. & BHAT, P. V. (1984). Cytoplasmic retinoic acid-binding protein in retinoic acid resistant human breast cancer sublines. *J. natn. Cancer Inst.* **73**, 793–799.

LOTAN, R., NEUMANN, G. & LOTAN, D. (1980). Relationships among retinoid structure, inhibition of growth and cellular retinoic acid-binding protein in cultured S91 melanoma cells. *Cancer Res.* **40**, 1097–1102.

MADEN, M. (1982). Vitamin A and pattern formation in the regenerating limb. *Nature, Lond.* **295**, 672–675.

MADEN, M. (1983). The effect of vitamin A on limb regeneration in *Rana temporaria*. *Devl Biol.* **98**, 409–416.

MARTH, C., MAYER, I. & DAXENBICHLER, G. (1984). Effect of retinoic acid and 4-hydroxytamoxifen on human breast cancer cell lines. *Biochem. Pharmac.* **33**, 2217–2221.

MATTHAEI, K. I., ANDREWS, P. W. & BRONSON, D. L. (1983). Retinoic acid fails to induce differentiation in human teratocarcinoma cell lines that express high levels of a cellular receptor protein. *Expl Cell Res.* **143**, 471–474.

MCCORMICK, A. M., NAPOLI, J. L. & DELUCA, H. F. (1978). High pressure liquid chromatographic resolution of vitamin A compounds. *Analyt. Biochem.* **86**, 25–33.

MEHTA, R. G., CERNY, W. L. & MOON, R. C. (1980). Distribution of retinoic acid-binding proteins in normal and neoplastic mammary tissues. *Cancer Res.* **40**, 47–49.

NIAZI, I. A. & SAXENA, S. (1978). Abnormal hind limb regeneration in tadpoles of the toad, *Bufo andersoni*, exposed to excess vitamin A. *Folia Biol. (Krakow)* **26**, 3–8.

ONG, D. E. & CHYTIL, F. (1978). Cellular retinoic acid-binding protein from rat testis. Purification and characterisation. *J. biol. Chem.* **253**, 4551–4554.

ONG, D. E., GOODWIN, W. J., JESSE, R. H. & GRIFFIN, A. C. (1982). Presence of cellular retinol and retinoic acid-binding proteins in epidermoid carcinoma of the oral cavity and oropharynx. *Cancer* **49**, 1409–1412.

ONG, D. E., PAGE, D. L. & CHYTIL, F. (1975). Retinoic acid-binding protein: occurrence in human tumours. *Science* **190**, 60–61.

PALAN, P. R. & ROMNEY, S. L. (1980). Cellular binding proteins for vitamin A in human carcinomas and in normal tissues. *Cancer Res.* **40**, 4221–4224.

PARAVICINI, U., CAMENZIND, M., GOWER, M., GIEGER, J.-M. & SAURAT, J. H. (1985). Multiple dose pharmacokinetics of Ro 10-1670, the main metabolite of etretinate (Tigason). In *Retinoids: New Trends in Research and Therapy* (ed. J. H. Saurat), pp. 289–292. Basel: Karger.

ROBERTS, A. B. & SPORN, M. B. (1984). Cellular biology and biochemistry of the retinoids. In *The Retinoids* (ed. M. B. Sporn, A. B. Roberts & D. S. Goodman), pp. 209–286. Orlando, Florida: Academic Press.

SANI, B. P. & HILL, D. L. (1986). A retinoic acid-binding protein from chick embryo skin. *Cancer Res.* **36**, 409–413.

SANI, B P., DAWSON, M. I., HOBBS, P. D., CHAN, R. L.-S. & SCHIFF, L. J. (1984). Relationship between binding affinities to cellular retinoic acid-binding proteins and biological potency of a new series of retinoids. *Cancer Res.* **44**, 190–195.

SCHINDLER, J., MATTHAEI, K. I. & SHERMAN, M. I. (1981). Isolation and characterisation of mouse mutant embryonal carcinoma cells which fail to differentiate in response to retinoic acid. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1077–1080.

SIEGENTHALER, G. & SAURAT, J. H. (1985). Cellular retinoid-binding proteins in normal and diseased human skin. In *Retinoids: New Trends in Research and Therapy* (ed. J. H. Saurat), pp. 168–174. Basel: Karger.

SLACK, J. M. W. (1983). Regional differences of protein synthesis in the limb regeneration blastema of the axolotl. In *Limb Development and Regeneration*, part A, pp. 557–563. New York: Alan R. Liss.

STOCUM, D. L. & THOMS, S. D. (1984). Retinoic-acid-induced pattern completion in regenerating double anterior limbs of urodeles. *J. exp. Zool.* **232**, 207–215.

SUMMERBELL, D. (1974). Interaction between the proximo–distal and antero–posterior coordinates of positional value during specification of positional information in the early development of the chick limb-bud. *J. Embryol. exp. Morph.* **32**, 227–237.

SUMMERBELL, D. (1983). The effect of local application of retinoic acid to the anterior margin of the developing chick limb. *J. Embryol. exp. Morph.* **78**, 269–289.

SUMMERBELL, D. & HARVEY, F. (1983). Vitamin A and the control of pattern in developing limbs. In *Limb Development and Regeneration*, part A, pp. 109–118. New York: Alan R. Liss.

THOMS, S. D. & STOCUM, D. L. (1984). Retinoic acid-induced pattern duplication in regenerating urodele limbs. *Devl Biol.* **103**, 319–328.

TICKLE, C. (1983). Positional signalling by retinoic acid in the developing chick wing. In *Limb Development and Regeneration*, part A, pp. 89–98. New York: Alan R. Liss.

TICKLE, C., ALBERTS, B. M., WOLPERT, L. & LEE, J. (1982). Local application of retinoic acid to the limb bond mimics the action of the polarizing region. *Nature, Lond.* **296**, 564–565.

TICKLE, C., LEE, J. & EICHELE, G. (1985). A quantitative analysis of the effects of all-trans-retinoic acid on the pattern of chick wing development. *Devl Biol.* **109**, 82–95.

TICKLE, C., SUMMERBELL, D. & WOLPERT, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature, Lond.* **254**, 199–202.

TROWN, P. W., PALLERONI, A. V., BOHOSLAWEC, O., RICHELO, B. N., HALPERN, J. M., GIZZI, N., GEIGER, R., LEWINSKI, C., MACHLIN, L. J., JETTEN, A. & JETTEN, M. E. R. (1980). Relationship between binding affinities to cellular retinoic acid-binding protein and *in vivo* and *in vitro* properties for 18 retinoids. *Cancer Res.* **40**, 212–220.

WANG, S.-Y. & GUDAS, L. J. (1984). Selection and characterisation of F9 teratocarcinoma stem cell mutants with altered responses to retinoic acid. *J. biol. Chem.* **259**, 5899–5906.

(*Accepted 22 May 1986*)