

Mechanism of skin morphogenesis. II. Retinoic acid modulates axis orientation and phenotypes of skin appendages

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Summary

The factors that determine the axial orientation and phenotypes of skin appendages were analyzed by studying the effect of retinoic acid (RA) on embryonic chicken skin explant cultures. With RA uniformly distributed in the culture media, the feather buds became smaller, were disoriented or were transformed into scale-like structures in a concentration-dependent manner (from 0.05 - 2.5 μM). With RA distributed as a gradient created by a RA-soaked anion exchange bead, a radial zone of inhibition with a rim of disoriented buds was observed. The new axis of the disoriented buds appeared to be determined by a combination of the original feather axis determining force and a new axial force pointing centrifugally away from the RA source. This observed result can be simulated with a computer model using a vectorial sum of different feather axial determination forces. The size of the inhibited zone is linearly correlated to the RA concentration and may be used to quantify the morphogenetic activity of retinoids. These effects are specific to developmental stages (Hamburg and Hamilton stage 31-34).

Both *all-trans* and *13-cis* RA have morphogenetic activity. Retinol has no effect and retinal has a small inhibitory effect but neither phenotypic transformation nor axial disorientation were observed. The antero-posterior gradient of homeoprotein *XIHbox 1* in feather buds became diffusive after RA treatment. RA dissolves dermal condensations and the distribution of N-CAM is altered from an anterior localized pattern to a diffusive presence in the bud cores. Endogenous retinoids in developing skins show developmental stage-dependent changes both quantitatively and qualitatively. The results suggest that RA either is or can modulate the endogenous morphogen(s) that determine the orientation and phenotype of skin appendages, and that this morphogenetic pathway involves Hox genes and adhesion molecules.

Key words: homeoprotein gradient, Hox, feather buds, scale, polarity, skin morphogenesis, retinoic acid, axis orientation.

Introduction

Pattern formation is a crucial event in embryonic development (summarized in Gilbert, 1991). This process requires cells to form ordered spatial arrangements and to differentially express genes at specific positions. Positional information mediated by some diffusible morphogen(s) has been proposed (Wolpert, 1978), but the nature of these hypothetical morphogens remains elusive. Recent progress has identified that homeobox (Hox) genes and retinoids are involved in the phenotype and axis determination during morphogenesis (Gehring, 1985; Tickle, 1991; De Robertis et al., 1991). For example, mutation of Hox genes causes transformation of appendages in *Drosophila*. Exogenous delivery of RA can induce the homeotic conversion of tail structures to legs in the marbled balloon frog (Mohanty-Hejmadi et al., 1992). RA can cause an antero-posterior reversal of axis in the developing nervous system of *Xenopus laevis* (Durstun et al., 1989; Ruiz i Altaba and Jessell, 1991) and in the spine of

mice (Kessel and Gruss, 1991). Localized application of RA in vivo by implanting a RA-soaked bead into the anterior limb bud can cause a mirror-image axis duplication in the limb digit pattern (Tickle et al., 1982). In the latter two cases, there are also corresponding changes in the homeobox gene expression patterns (Nohno et al., 1991; Izpisua-Belmonte et al., 1991; Kessel and Gruss, 1991), implying a series of molecular events involving retinoid-related molecules and Hox genes along the pattern formation pathway.

One of the classical models in the study of pattern formation is the induction of skin appendages. Studies of morphological and biochemical events in the various stages of development of feathers, hairs and scales have been described (Lucas and Stenheim, 1972). The central concern is to determine how genes are locally regulated to form many different types of skin appendages from the genomic DNA of a single organism. A variety of epithelial-mesenchymal recombination experiments have been performed which led to the conclusion that the message for the phenotype of skin

appendages is stored in the mesenchyme (reviewed in Sengel, 1976). However, the molecular basis of this stored message remains unknown.

We have recently discovered that there are anterior-posterior (A-P) homeoprotein gradients present transiently in developing feather buds (Chuong et al., 1990). This and other's previous results (reviewed in De Robertis et al., 1991) led us to hypothesize that homeoproteins may be involved in setting up the A-P axis of cell fields at different levels, first for the body axis, then for the limb axis, and finally for the feather axis. With further characterization, we have shown that there are position-specific expression patterns of homeoproteins (Chuong, 1991). If this hypothesis is correct, alteration of Hox genes should cause changes in the orientation or phenotypes of skin appendages.

RA has been shown to regulate homeobox gene expression both *in vitro* (Simeone et al., 1990) and *in vivo* (Nohno et al., 1991; Izpisua-Belmonte et al., 1991; Kessel and Gruss, 1991) and the human homeobox 3D promoter has been shown to be regulated by RA in embryonal carcinoma cells (Alcioni et al., 1992). Therefore in the present study, we exposed developing feather buds to RA in culture media or a local micro RA gradient. We observed alterations of orientation and phenotypes of skin appendages concomitant with alterations of the distribution of homeoprotein and N-CAM expression.

Material and method

Chicken embryos

Fertilized White Leghorn chicken eggs were obtained from Red Wing Farm (Los Angeles) and incubated in a humidified chamber at 38°C (Humidare). The embryos were staged according to Hamburger and Hamilton (1951).

Skin explants culture

White Leghorn chicken embryos between 6.5 (stage 30) and 8 (stage 34) days of incubation were obtained from K and R Farm (Westminster, CA). The dorsal skins between the lower neck to the tail were dissected in Hank's buffered saline solution (Gibco/BRL) under dissection microscope and placed on culture inserts in 6-well culture dishes (Falcon). The culture media contains DMEM with 2% fetal calf serum and was changed every two days. The cultures were incubated at 37°C at an atmosphere of 5% CO₂ and 95% air. The morphogenesis of feather buds were recorded with Olympus OMT 4 camera using inverted microscope (Olympus IMT-2) or dissection microscope.

Immunostaining

Immuno-alkaline phosphatase stainings were performed on paraffin sections as described in Chuong et al. (1990). At days to be fixed, the skin explants were fixed with Bouin's fixative for 1 hour, and washed overnight in 70% ethanol. The samples were dehydrated through a series of ethanol washes, cleared in xylene, and embedded in paraffin. Tangential or longitudinal sections at 6 µm were collected on gelatin treated slides. After de-paraffination, the sections were incubated with primary antibodies overnight in a humidified chamber at room temperature, followed by treatment of alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega) at 1:700 dilution for 3 hours at room temperature. NBT and BCIP

(Promega) were used as substrates in which a positive reaction results in purple staining. The sections were mounted in glycerin mounting media.

Antibodies to N-CAM were described in Chuong and Edelman (1985). Polyclonal antibodies to XIHBox 1 were produced by immunizing with rabbit fusion proteins (Oliver et al., 1988) and its expression pattern in chicken feather development was described in Chuong et al. (1990). We will tentatively call the chicken protein(s) recognized by anti-Xenopus XIHbox 1 as cXIHbox 1. DNA sequence data so far suggests that XIHBox 1 most closely resembles mouse Hox 3.3. Whether cXIHbox 1 is indeed one or several homeoproteins is unknown, but the distribution pattern of cXIHbox 1 provides a useful markers for homeoprotein distribution. Antibodies to alpha and beta keratin were kindly provided by Dr. R. H. Sawyer and L. Knapp and was performed according to O'Guin et al. (1982) and Zeltinger and Sawyer (1992).

Retinoid

Retinoids including all-*trans* RA, 13-*cis*-RA, retinol and retinal were obtained from Sigma. Retinoids were weighed and dissolved in dimethyl sulfoxide (DMSO) to make 10 mg/ml stock solution. They were aliquoted into 20 µl aliquots, wrapped in aluminum foil and stored at -40°C. One aliquot was thawed immediately before use and discarded in the same day. For experiments using retinoid in culture media, aliquots of stock solution were added to the media to make various final concentrations (0.05 - 9.0 µM). In some experiments, absolute ethanol was used instead of DMSO, and the results were the same. For preparation of retinoid beads, we followed Tickle et al. (1982) with some modification. AG1-X8 ion exchange beads (Bio-rad) of 100-200 µm diameter were soaked for 2 hours in either DMSO only or in solutions containing 0.01 - 5 mg/ml of retinol, retinal, all-*trans* RA, or 13-*cis*-RA. The beads were then washed in DMEM and placed on skin explant cultures using micro-manipulator (Narishigi) under dissection microscope. Cultures containing retinoids were kept away from light as much as possible.

Scanning electron microscopy

Skin explant cultures intended for scanning electron microscopy were fixed in 1/2 strength Karnovsky's fixative overnight at 4°C, post-fixed in thio-carbohydrazide, and followed by osmium fixation. The specimen were critically pointed dried and coated with gold palladium. They were viewed with an Hitachi S-570 scanning microscope. We thank Dr Janet Blanks and Ms Chris Spee of the Doheny Eye Institute for their expertise and help in processing these samples.

Computer model

We have generated a computer model to simulate the effects of RA on feather axis determination. This model was written under the premise that RA supplies an axial force which radiates out from the central bead in a concentration-dependent manner. In this model, the axial vector provided by the RA bead is added to the endogenous axis generating vector to determine the predicted feather axis. Please see the legend of Fig. 9 for detail.

Results

Dorsal skin from stage 33 - 34 chicken embryos were maintained for up to 7 days as organ cultures. The beginning explant already contained small dermal condensations (Fig. 1A) which developed into elongated and conically shaped

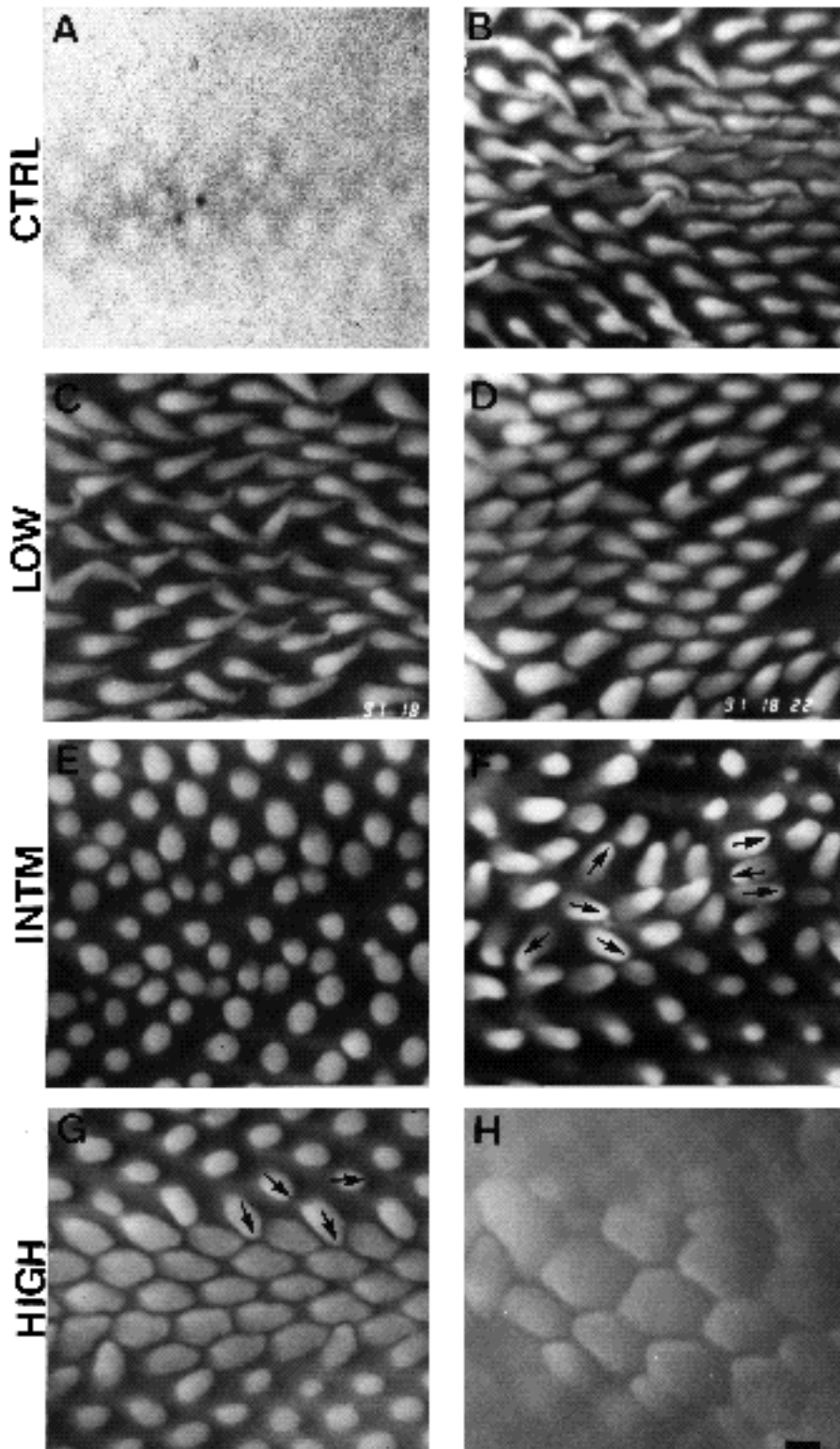


Fig. 1. Effect of RA in media on embryonic chicken skin explant cultures. (A) Dorsal skin explant from stage 33/34 chicken embryo at day 0 of culture. (B) Control (CTRL) after 6 days of culture. (C, D) Cultures in the presence of 0.05 - 0.75 μM RA (LOW) in media showing no apparent effect or slow growth of feather buds. (E, F) Cultures in the presence of 1 - 1.61 μM RA (INTM) in media showing slow growth or disorientation of feather buds. (G, H) Cultures in the presence of 2 - 2.5 μM RA (HIGH) in media showing transformation into scale-like structures. Arrows = orientation of feather buds. Bar = 200 μm .

feather buds in 6 days (Fig. 1B). In these cultures, both body axis and feather bud axis were oriented so that the anterior end was pointing towards 9 o'clock and the posterior end towards 3 o'clock. When the antero-posterior axis pointed to 3 o'clock, it was designated as 0°. When this axis pointed between 2 and 9 o'clock, it was designated as +30° to +180°.

When this axis pointed between 4 and 9 o'clock, it was designated as -30° to -180°.

Effect of RA in media

To determine the effect of RA on skin morphogenesis, RA

Table 1. Effect of RA in media on skin explant cultures

Stage	RA conc. (μM)	n*	Incidence of phenotypes (%)				
			No effect	Slow growth	Disorientation	Scale like	Inhibition
33-34	0.05-0.75	9	67	33	0	0	0
	1.00-1.61	29	14	50	29	14	4
	2.00-2.50	17	0	24	24	53	6
	3.00 or more	3	0	0	0	0	100

*Number of total skin explant cultures.

was added to the media of embryonic chicken skin explant cultures. Vehicle alone (DMSO or ethanol at the same concentration as the experimental ones) had no effect. At low dosages (0.05 - 0.75 μM) of RA, there was no effect in most (67%) of the explants (Fig. 1C), and 33% of the explants in this range of RA treatment exhibited small and short feather buds (Fig. 1D; Table 1). With intermediate dosage (between 1 and 1.61 μM) of RA, we observed a higher frequency of small feather buds (Fig. 1E; Table 1) and began to see many buds showing random orientations, some with axes pointed perpendicular or opposite to the normal axis (Fig. 1F; Table 1). High RA dosage (between 2 and 2.5 μM) treatment transformed feather buds into skin appendages morphologically very similar to the scale (Fig. 1G, H; Table 1). This similarity can be appreciated by comparing Fig. 1E and 8C, whole-mount and longitudinal sections of RA-converted feather buds, with reticulate scales shown in Fig. 2b and 1d of Zeltinger and Sawyer (1992). Above 3 μM RA, the feather buds appeared to be totally inhibited.

To analyze further the morphological changes, we used scanning EM to study the RA-treated explants. The well-developed and elongated feather filaments (Fig. 2A) were seen to become shortened and flattened in the presence of 1.5 μM RA (Fig. 2B). Many of them can be seen to point between 0 and -90° and some pointed perpendicularly to the original axis. In some regions, the polarity was completely lost and the original feather buds appeared to be very similar to scutate scales (Fig. 2C).

To quantify the changes of orientation, we measured the axial orientation of all the buds on the explant from which Fig. 1F was taken. The results showed a nearly even distribution of axial orientation in all directions in contrast to the control cultures in which the axes of feather buds pointed between $\pm 20^\circ$ (Fig. 3).

The effects of RA are developmental stage-dependent. When younger skins were used, feather development was inhibited completely by RA even at lower concentrations than those reported above. When older skins were used, no effects were observed even at higher concentrations than the reported concentrations. The effect is also specific to RA. Similar effects were observed whether RA was dissolved in absolute ethanol or DMSO. Equivalent amounts of the ethanol or DMSO carriers added to the media had no effect. For other retinoids, we used retinol (1.0 - 3.0 μM) and retinal (1.0 - 3.0 μM) which showed no apparent effect. Above 3 μM , retinol, retinal or RA alone appeared to be toxic and inhibited the bud growth completely.

Effect of RA gradient

The randomization of feather bud orientation is intriguing. To analyze the precise role of RA on feather bud orientation, we created a local RA gradient on skin explant cultures using AG1-X8 anion exchange beads soaked in retinoid as originally designed by Tickle to study limb axis (Tickle et al., 1985). The beads however are too big to be implanted inside the dermal condensations which are only about 100 μm in diameter at this stage. We had to place them on the surface of the explant. In control experiments, we observed that neither the beads themselves nor dimethyl sulfoxide (DMSO)-soaked beads had a mechanical or steric hinderance effect on feather bud development, except a very slight displacement of feather buds if the beads happened to be immediately next to the buds (Fig. 4A).

In contrast, when RA-soaked beads (Fig. 4B) were used, we observed very interesting results. Regions immediately next to a RA-soaked bead were completely inhibited. Toward the periphery of the inhibitory zone, there were small, round feather buds. On the margin, a rim of the buds with deflected axes was observed (arrows). Anterior to the beads (towards the 9 o'clock direction), the buds pointed to $+80^\circ$ and -80° , while posterior to the beads (towards the 3 o'clock direction), the buds did not change orientation. Above and beneath the beads, the buds showed a trend of gradually decreasing deflection angles from the left to the right (shown by arrows in Fig. 4B). The elliptical shape of the inhibitory zone was due to the cumulative effect of the 4 beads placed in a row. When a single bead was used, a similar alteration of pattern was observed except the inhibitory zone was round. Both all-*trans* RA and 13-*cis* RA can alter the orientation of feather buds (Table 2). Retinol beads at the same or higher concentration had no effect (Fig. 4C). Retinal beads showed no effect (83%) or a small inhibitory zone (17%). However, no alteration of feather bud axis was observed (Table 2).

To explore the effect of RA on dermal condensations, we did a time course study in which we photographically recorded explants every two days. When RA beads were placed on the explant at stage 31, the dermal condensations had just formed and were small yet visible (Fig. 5A). Normal condensations grew steadily in size and were visible by day 2. The condensations became round buds at day 4 and became elongated buds at day 6 (Fig. 5B - D, blank arrows). The dermal condensations immediately adjacent to a RA-soaked bead (about 200 μm diameter away from the center of beads) disappeared after two days in culture. The dermal condensations at about 400 μm distance became more and

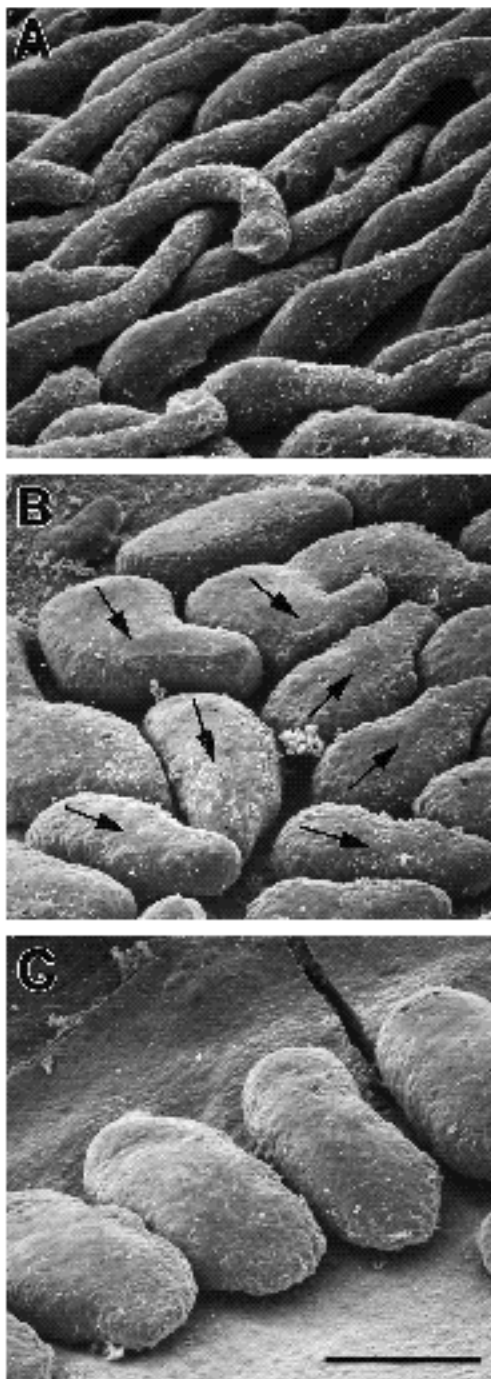


Fig. 2. Scanning EM of RA-treated skin explants. Stage 33 cultures cultured for 7 days. (A) Control showing well-developed feather filaments. (B, C) Explants cultured in the presence of 1.5 μM showing disoriented and flattened feather buds (B) and scutate scale-like structures (C). Arrows = orientation of feather buds. Bar = 200 μm .

more diffusive from day 2 to day 4 and eventually disappeared at day 6 (Fig. 5 A - D, asterisks). The dermal condensations at about 700 μm distance appeared similarly at day 2, became smaller at day 4, and caught up in size at day 6 but with altered orientation (Fig. 5A - D, arrows). Therefore RA appeared to be able to either repress or modulate the formation of dermal condensations.

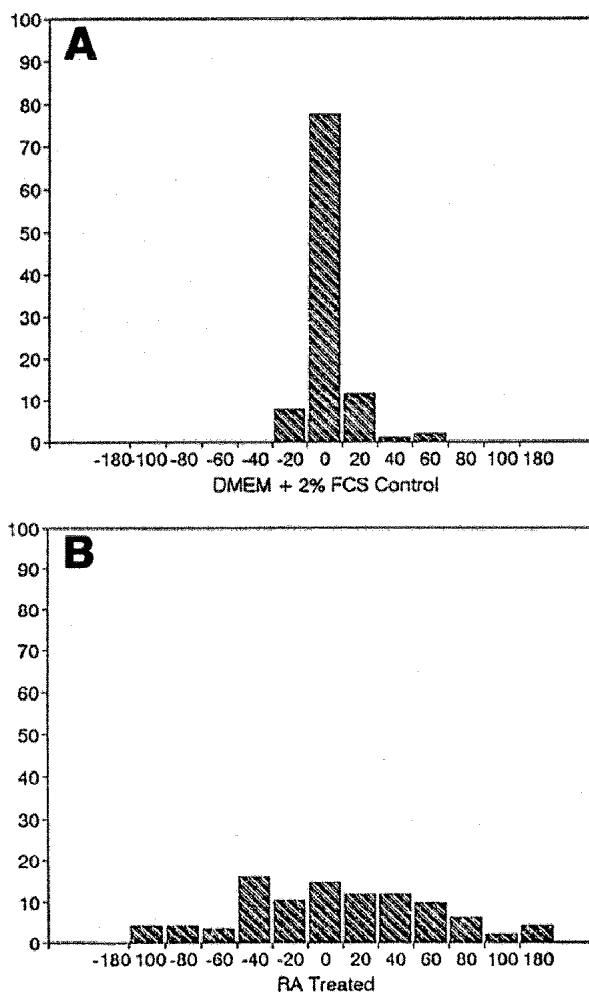


Fig. 3. Random axial orientation of RA-treated feather buds. X axis, the orientation of feather bud axis. Please see the beginning of results for the definition of orientation. Y axis, the frequency of buds with a certain orientation. Stage 34 skins cultured for 4 days showed feather bud orientation distributed between -20° to $+20^\circ$ (A). Cultures with 1.5 μM RA (B) showed random orientation. More than 100 buds were measured from pictures of the whole explant.

The effects of RA concentration were also studied. Beads were soaked in 0.01 - 5 mg/ml RA and placed on stage 31 skin explant. At 0.01 mg/ml, there was no apparent effect of RA. At 0.5 mg/ml, there was a small inhibitory zone. Above 1 mg/ml, the inhibitory zone enlarged and we began to observe disorientation of feather buds. In between the complete inhibited zone and the disoriented buds were small round buds (Fig. 6A - C). The incidence of different phenotypes at different concentrations of RA on the ion exchange beads is summarized in Table 3. The diameters of the inhibitory zones were measured and plotted against the RA concentration showing a linear relationship between these two parameters (Fig. 6D).

Alterations of other molecules

We have previously shown that N-CAM is enriched in the anterior feather buds (Chuong and Edelman, 1985; Fig. 7A)

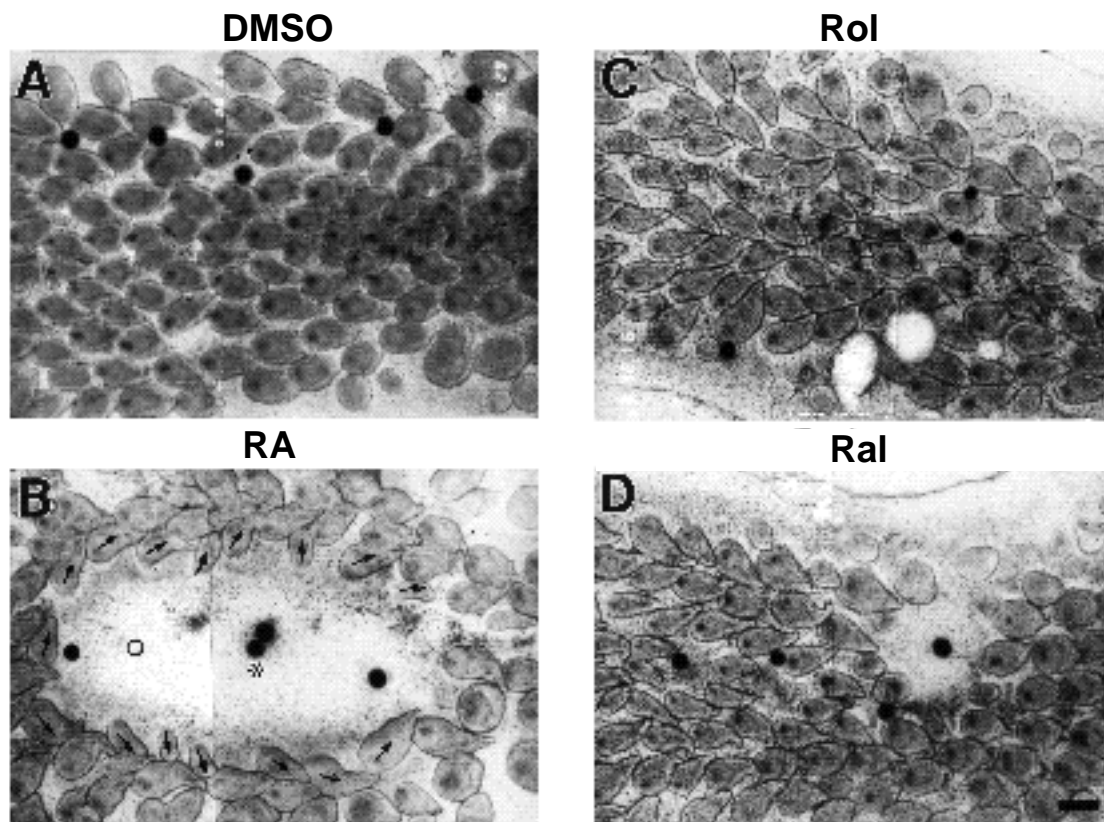


Fig. 4. Effect of retinoid-soaked beads on feather axial orientation. Stage 31 skin cultured for 6 days with retinoid-soaked beads (asterisk) placed on the explant. (A) Culture with beads soaked in DMSO showed no effect. (B) Culture with beads soaked in all-*trans* RA (1 mg/ml) showed a zone of inhibition with the buds at the rim showed deflected axis orientation, suggesting the existence of axial forming force radiating away from the RA source. (C) Beads soaked in 2 mg/ml retinol (Rol) showed normal growth and orientation. (D) Beads soaked in 2 mg/ml retinal (Ral) appeared mostly normal but small inhibition zone was observed but no change of bud orientation was observed. Arrows = feather bud axial orientation. Asterisk = beads. The bead on the left of panel B moved during fixation, the original position is indicated by open circle. Bar = 200 μ m.

and that antibody to N-CAM perturbed dermal condensation formation (Jiang and Chuong, 1992). Because RA appeared to influence the process of dermal condensation, we examined the distribution of N-CAM in all-*trans* RA-treated feather buds. For the skin explants cultured for 5 days, N-

CAM was diffusely distributed and concentrated more in the core and base of the buds (Fig. 7B). We also examined homeoprotein expression pattern in these 5 day explants. We have earlier shown that the chicken protein or proteins which immuno-cross reacted with antibodies to XIHbox 1 (desig-

Table 2. Effect of retinoid-soaked beads on skin explant cultures

Stage	Type of retinoid*	n†	Incidence of phenotypes (%)			
			No effect	Small Round buds‡	Disorientation	Inhibition
31	All- <i>trans</i> RA	17	0	46	68	100
	13- <i>cis</i> -RA	8	25	13	88	88
	Retinal	12	58	25	0	17
	Retinol	4	100	0	0	0
	DMSO	10	100	0	0	0

*For this set of experiments, the beads were soaked in 1 mg/ml retinoids.

†Number of total beads implanted.

‡In these experiments, it was difficult to differentiate the slow growth and scale-like buds as listed in Table 1. They were pooled in this "small and round buds" category.

Table 3. Effect of beads soaked in different concentrations of RA on skin explant cultures

Stage	RA beads conc. (mg/ml)	n*	Incidence of phenotypes (%)			
			No effect	Small round buds	Disorientation	Inhibition
31	0.01	4	100	0	0	0
	0.10	4	0	100	0	100
	0.50	4	0	100	0	100
	1.00	28	0	46	68	100
	2.00	17	0	59	53	100
	3.00	6	0	33	67	100
	4.00	5	0	0	100	100
	5.00	5	0	0	100	100
34	1.00	6	50	0	50	100
	2.00	6	0	100	50	100

*Number of total beads implanted.

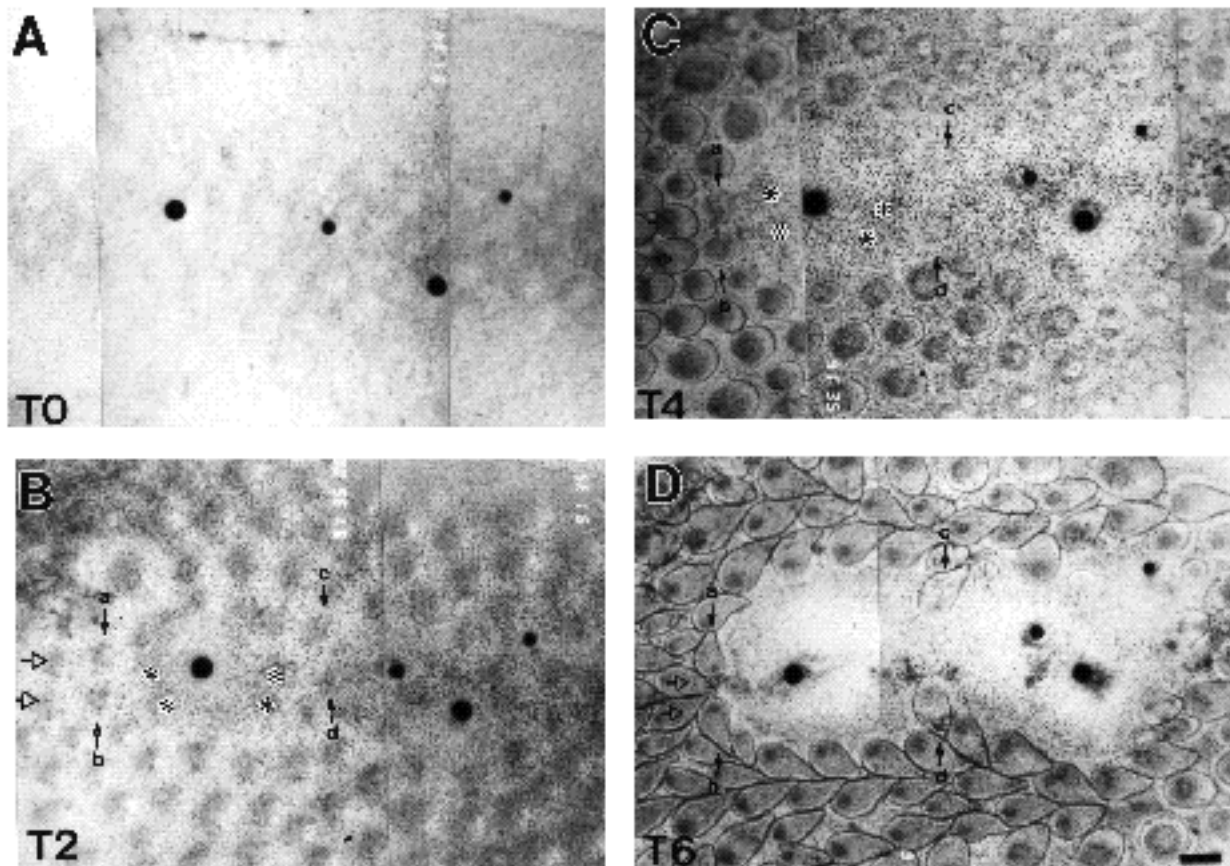


Fig. 5. Time course of changes in dermal condensations by RA beads. Stage 31 skin explant cultured for the time (T) of 0 (A), 2 (B), 4 (C) and 6 (D) days with 4 beads pre-soaked in 2 mg/ml 13-*cis* RA. For each panel, it is the same piece of explant. Arrows a-d = condensations of four feather buds that turn almost 90°. Asterisk = four condensations that gradually disappear and became part of the inhibited zone. Open arrow = normal feather buds. Bar = 200 μ m.

nated as cXIHbox 1 here) is distributed in an antero-posterior gradient in feather buds (Chuong et al., 1990) and has a body position-specific expression pattern (Chuong, 1991). We have shown that in explant cultures the expression of both cXIHbox 1 and Hox 5.2 in untreated controls were the same as in ovo studies. Anti-XIHbox 1 staining of control feather buds from the mid-portion of the explant showed an antero-posterior gradient (Fig. 7B) as described. In contrast, after growth in the presence of RA, buds in a corresponding position showed much more diffuse cXIHbox 1 staining pattern (Fig. 7D).

We then examined changes in the explant cultured for as long as 8 days. In the control explants, NCAM was present in the dermal papilla at the base of forming feather filament, dense mesenchyme surrounding the follicle sheath and depressor muscle. The original asymmetry still could be seen but will soon disappear. Alpha keratin was present on the epidermis (Fig. 8A-E). After RA treatment, feather buds did not invaginate into skin to form follicles, nor do they elongate to form feather filaments. The feather bud became short scale-like stump. N-CAM was present in the feather bud mesoderm, but no dermal papillae formed. The N-CAM-positive mesodermal cells appeared to be more tightly packed than N-CAM-negative cells (Fig. 8C, arrows, cell group margin indicated by broken line). In normal developing scale, N-

CAM staining pattern has been shown to be more diffusive than those in the feather bud (Shames et al., 1991). Alpha keratin remained positive in epidermis (Fig. 8A-E). Staining with beta keratin was negative in both control and RA-treated explants (not shown). It can also be seen from phase contrast that cells treated with this dose of RA for 8 days remained healthy (Fig. 8C, C) and were capable to express alpha keratin (Fig. 8E, E). The sections also showed that these altered feather buds had morphology similar to reticulate scales (Zeltinger and Sawyer, 1992). In the sections of RA-treated explants, no gland-like structures were observed as reported in RA-treated mouse whisker (Hardy et al., 1990).

Discussion

Retinoids are considered a possible morphogen during embryonic development and over-exposure to retinoids has been shown to have teratogenic effects (reviewed in Lammer et al., 1985). In this study, we examined the effects of retinoids in feather morphogenesis. We showed that RA can modulate the pattern formation process and exposure to RA caused alterations of phenotypes and axial orientation of skin appendages. Polarized distribution of cXIHBox 1 (Chuong et

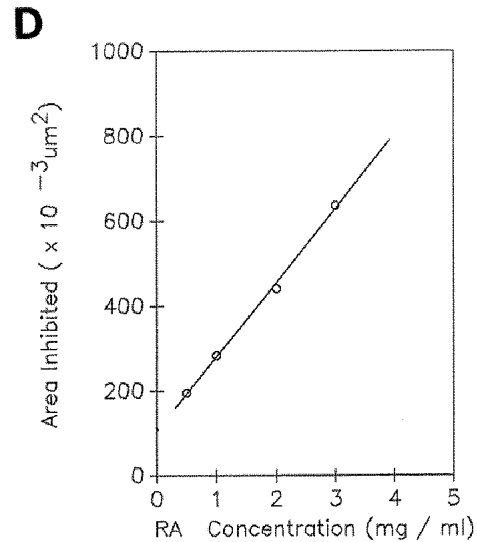
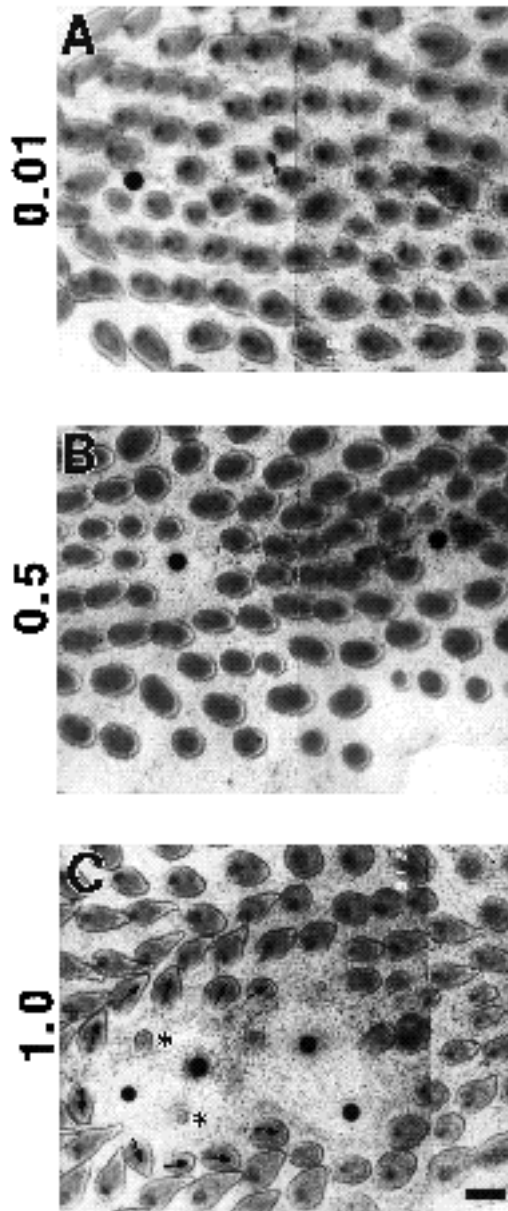


Fig. 6. Effects of beads soaked in different concentrations of RA on skin explants. Beads were soaked in 0.01 (A), 0.5 (B) or 1 mg/ml (C) all-*trans* RA and placed on stage 31 skin explants which was cultured for 6 days. Note there is no effect in A, small inhibitory zone in B, and larger inhibitory zone with small round buds (asterisks) and altered feather bud orientation (arrows) in C. (D) Area of inhibitory zone (assuming the zone is round) plotted against RA concentration showed a linear relationship. Bar = 200 μm .

al., 1990) and N-CAM (Chuong and Edelman, 1985) became diffusive in affected feather buds. These results suggest that RA either is, or can modulate the endogenous morphogen(s) which determine the phenotype and specify the axis of skin appendages.

Effect of RA on phenotype determination of skin appendages

Skin appendages start as a flat sheet of ectoderm which interacts with the underlying mesoderm. After induction, the differentiation state of the induced placode epithelia is altered and it has the potential to become hairs, whiskers, glands, feathers, scales, etc. (Sengel, 1976). Hardy et al. (1990) have hypothesized that morphogenesis of skin appendages can be divided into at least two stages: an induction stage during which the decision to initiate a skin

appendage is made, followed by a specification stage during which the specific phenotype of the skin appendage is determined. In our experiment, we observed the largest spectrum of converted phenotypes when RA was added at a time after small dermal condensations had already formed (stage 33-34, Table 1). If RA was added earlier than stage 31 at the time dermal condensations were just forming, most skin appendages were completely inhibited and a flat explant was obtained. If RA was added after stage 34, no effect on the skin appendages were observed. Thus RA may have to act on the specification stage (Hardy et al., 1990) to have the effect observed in this report. The RA concentration also has an effect on phenotype determination. At concentrations lower than 0.5 μM RA had no apparent effect. Above 3 μM RA had completely inhibited feather bud development which may be due to a toxic effect. Within this range, we observed slow growth, axial disorientation, scale-like morphology in a con-

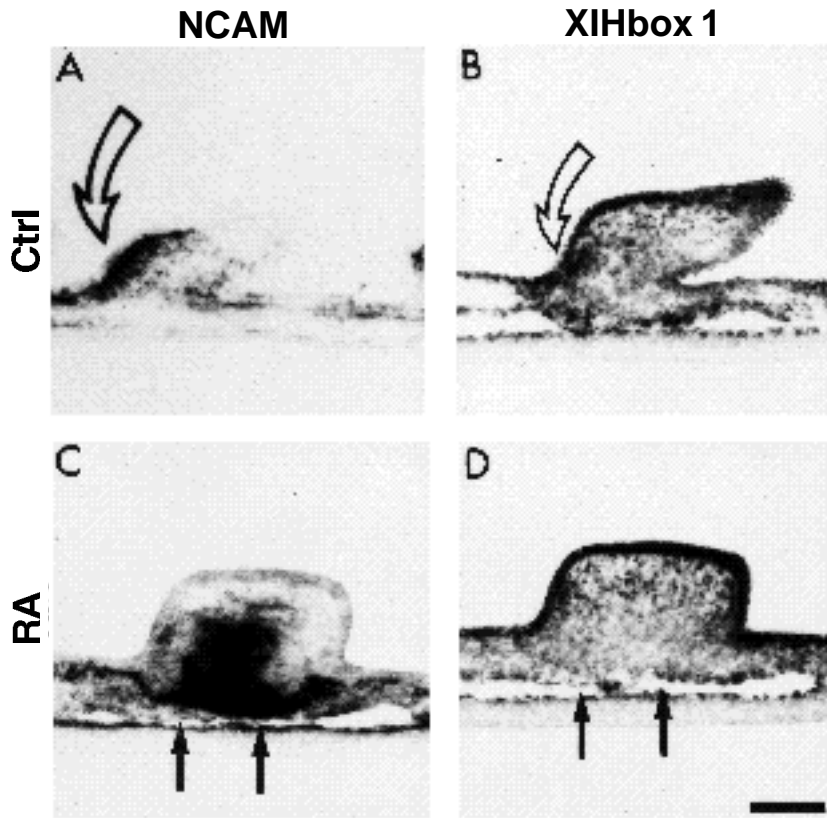


Fig. 7. Alteration of N-CAM and cX1HBox 1 in all-*trans* RA-treated skin explants cultured for 5 days. Stage 33 skin explant cultured without RA (A, B) or with 2 μ M RA in media (C, D) were sectioned along the anterior - posterior axis of explants and stained with anti-N-CAM (A, B) and anti-X1HBox 1 (C, D) followed by alkaline phosphatase-conjugated secondary antibodies. Note both N-CAM and cX1HBox 1 are enriched in anterior mesoderm of feather buds (curved arrow). N-CAM is absent on epidermis whereas cX1Hbox 1 is also present in the epidermis. After RA treatment, the feather buds express a scale-like morphology and the staining of both N-CAM and cX1Hbox 1 have become very diffusive (straight arrows). Bar = 100 μ m.

centration-dependent manner (Figs 1, 2). Thus, the effect of RA on phenotype determination of skin appendages appears to be concentration- and developmental stage-dependent.

While RA can transform developing feather buds into scale-like structures, it can also transform developing scales into feathery scales (Dhouailly et al., 1980; Fisher et al., 1988; our unpublished data). In mouse, vitamin A transformed hair follicles into glands (Hardy, 1968). These experiments showed that in general skin appendage phenotypes can be interconverted if the morphogenesis pathway is diverted in the specification stages of skin appendage formation. RA was shown to be able to convert cervical vertebrae and lumbar vertebrae into thoracic vertebrae (posteriorization and anteriorization respectively, Kessel and Gruss, 1991). Our observations suggest that RA can convert feather buds into skin appendages with morphology similar to that of scale. Whether the effect of RA is mediated through epithelium or mesenchyme is unknown, and we are currently using epithelium / mesenchymal recombination cultures to address this issue experimentally.

During evolution, a variety of skin appendages such as reptilian scale, bird feather and scale and mammalian hair have evolved. The messages of induction can cross-talk between different species (Sengel, 1976) and it appears that ectoderm first receives message(s) to make a skin appendage then decides what type of skin appendage to make (Hardy et al., 1990). Therefore it is compelling to speculate that new types of skin appendages may have evolved through re-setting homeobox codes of skin appendages (Chuong, 1991) possibly by abnormal retinoid metabolism. Since altered skin appendage phenotypes are not lethal, a large variety of skin appendages have evolved in birds.

Effect of RA on axis specification of skin appendages

The most intriguing RA effect is the alteration of the feather bud axis (Fig. 1F and Fig. 3). To analyze precisely the disorientation effect, we created a minute RA gradient using anion exchange beads originally used to study limb development (Tickle et al., 1982). Skin explants treated with these beads showed a dose response to RA in which three threshold zones can be distinguished. The first zone is a region in the immediate vicinity of the RA-soaked beads (about 200 μ m radius when beads were treated with 2 mg/ml 13-*cis* RA) and all skin appendages are inhibited. The second zone is a concentric ring (between 200 and 300 μ m in radius using the same example) containing small round buds. As the distance from the bead increased and the concentration of RA decreased, a rim of developing feather buds (about 400 μ m from the center of the bead, or about 40 cells away from the RA source because the diameter of these dermal cells are about 10 μ m) develop with deflected axial orientation. Outside this third zone, feather buds develop normally. (Figs 4, 5 and 6). This phenomena may be explained in the most simplified way by the gradient model (Wolpert, 1978) which suggests that a diffusible morphogen can specify different patterns at different threshold values of that morphogen. Here RA can be the morphogen itself or modulator of the endogenous morphogen.

Further analyses of the degree of alteration showed that the new feather axial orientation depends on its distance and position relative to the RA source. Analyses of the deviated angle of feather axes suggests that the difference between the altered and original axis can best be explained by the existence of a new axial forming force pointing centrifugally away from the RA source acting in conjunction with the endogenous axis determining factor. A computer program

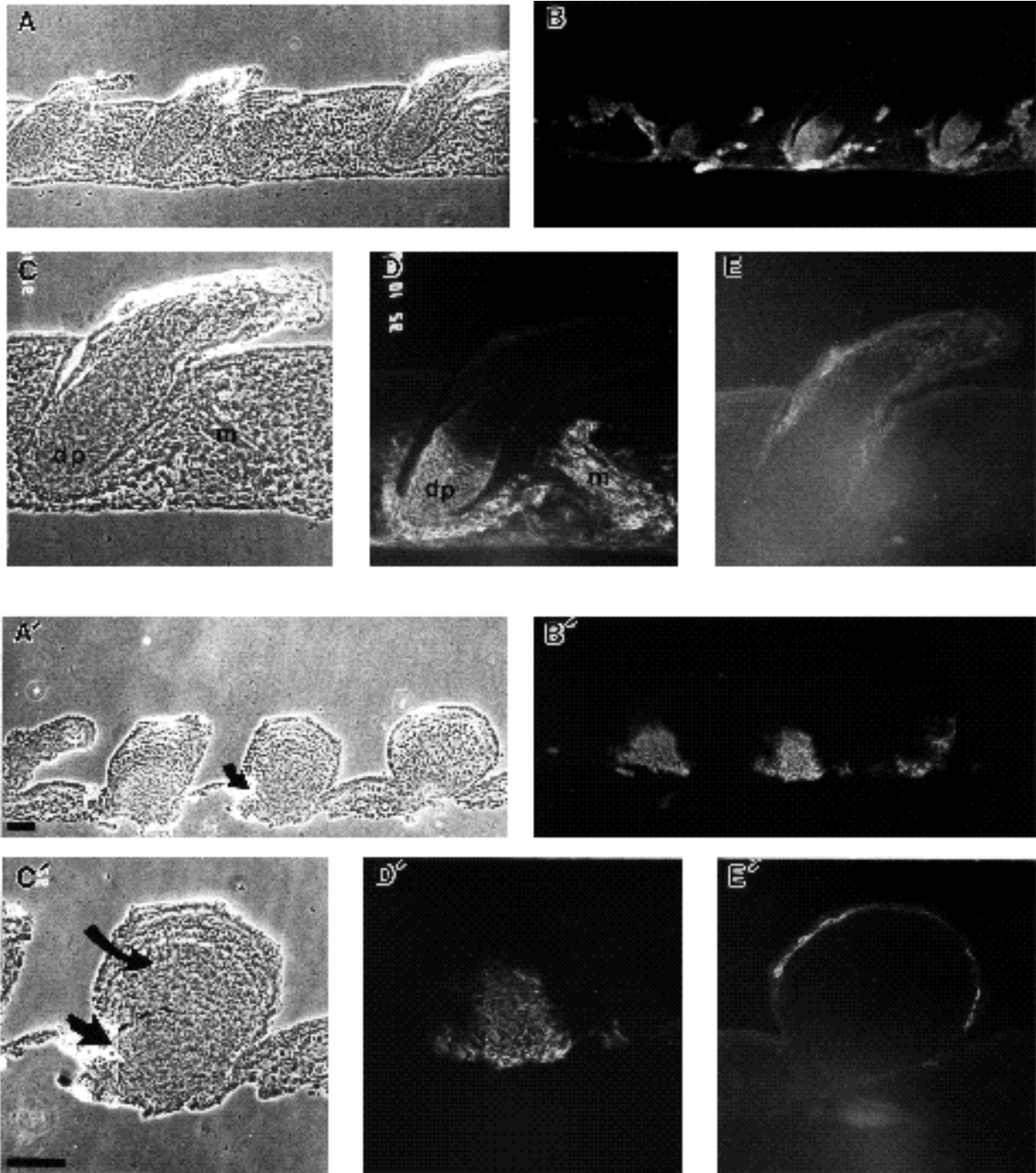
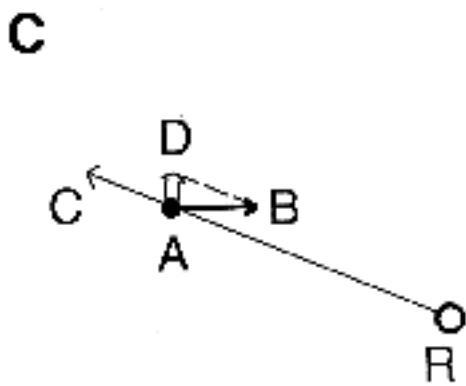
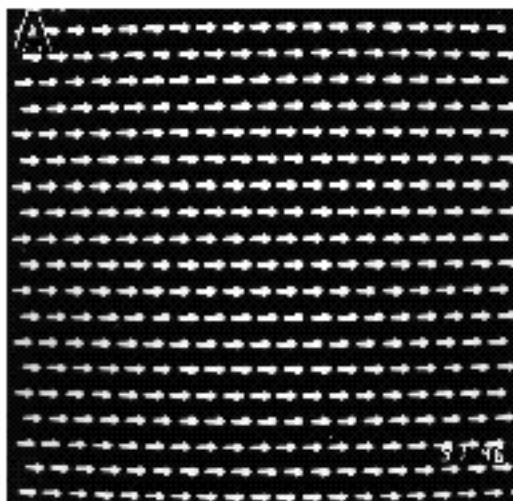


Fig. 8. Alteration of N-CAM and keratin in RA-treated skin explants cultured for 8 days. Stage 34 skin explant cultured without RA (A-E) or with 2 μ M RA in media (A' - E') were sectioned along the anterior - posterior axis of explants and stained with anti-N-CAM (B, B', D, D') and alpha keratin (E, E') followed by fluorescein-conjugated secondary antibodies. A, A', C, C', phase contrast pictures; the rest, fluorescent pictures. dp, dermal papilla; m, depressor muscle. Straight arrow, N-CAM-positive mesodermal cells were more tightly packed. Curved arrow, N-CAM-negative cells appeared to be less tightly packed. Broken line, separation of these two cell groups. Note both normal and RA-treated explant express N-CAM and alpha keratin but the distribution patterns were altered. Also note cells in RA-treated buds were not pyknotic or obviously sick (C and C'). Bar, 100 μ m.

was therefore designed which performed the vector sum of these two forces. In this computer model, the new orientation is hypothesized to be the vector sum of the original skin appendage direction vector and the orientation modulating

vector derived from the RA bead. The modulating vector radiates away from the bead, while its intensity decreases inversely with distance ($1/d^x$). For Fig. 9, it was arbitrarily chosen as $1/d^2$, assuming that diffusion is the major mecha-



nism. When the concentration is above a designated threshold, total inhibition of skin appendages was assigned. (Computer program is available upon request). The computer simulated pattern is very similar to the experimentally altered feather pattern (Fig. 9; please compare with Figs 4B, 5D, 6C). The effect of distance, which mainly represents a change in the concentration of the morphogen (Fig. 6), is rather steep and could be explained either by the abrupt threshold response of feather germs to RA or by a rapid decrease of the RA gradient due to the combination of diffusion and convection in the culture condition. (The beads were

Fig. 9. Computer model showing the skin appendage axis altered by a point source of the “morphogen.” (A) Control. Each arrow represents a hexagonally arranged skin appendage axis. (B) When a bead with “morphogen” capable of modulating the axis orientation was placed in the center of the skin appendage field, the orientation of the skin appendage was altered. The angle of deflection depends on the relative position of a particular feather bud and the morphogen source. Towards the left of the bead, the angle is deflected more, while towards the right of the bead, the angle is not altered. (C) Illustration showing how we wrote this computer program. The program is based on the following formula

$$|AC| = K \frac{[R]}{|RA|^n}$$

R, source of morphogen capable or modulating feather axis, in this case, it is RA.

[R], concentration of morphogen in the source. In this case, it reflects the amount of RA adsorbed on the anion exchange bead AG1-X8.

A, the location of a certain feather germ.

AB, the original feather axis forming force, shown in thick arrow.

RC, a hypothetical new modulating axial forming force pointing centrifugally away from R, shown in thin arrow.

AC, the axis modulating force acting on feather germ A.

|RA|, distance between morphogen source and a feather germ.

n, power with which the strength of morphogen decrease. In our program, one can designate any number. In the figure shown, we assumed that RA diffuses within the feather explant plane and assigned a value of 2.

Inhibitory zone: we assigned a RA value over which all feather germs are inhibited.

K, constant.

It is obvious $BD = AC$. We then perform vector sum

$$AB + BD = AD$$

AD shown in open arrow is the new axis of the disoriented bud.

Using this program, we can place RA bead at any position on a plane of hypothetical feather germs arranged in hexagonal pattern. The result of the computer generated image is amazingly similar to the experimental results shown in Figs 4B, 5D and 6C.

situated on top of the explant which was covered by a thin layer of media.) The exquisite pattern of the gradual rotation of feather axes also suggests that what we have observed is not just random teratogenic effect but rather an ordered modulation acting on normal morphogenic process.

The data here showed that an exogenously added RA gradient can modulate the feather bud axis. Whether there are endogenous RA gradients in developing skin remains undetermined. If we assume there is a RA gradient with the concentration of RA decreasing in the direction in which the feathers point, the feather buds anterior to the bead will be exposed to a reversed gradient and the orientation will be altered. The bud posterior to the beads will be exposed to a gradient that runs in the same direction as the presumptive endogenous gradient and the orientation will not be altered. The results that we observed are consistent with these presumptions. Another point is that with the geometry of the feather pattern, if there is a macrogradient running across the body axis, the absolute concentrations of RA in adjacent

feather buds will differ. In this case, the major parameter may be the relative difference of RA concentration across the individual bud (microgradient). Presumably it is such a microgradient that would influence the local expression of homeobox genes and adhesion molecules. From our work on homeoprotein distribution in feather buds, the results suggest that there is a macro homeoprotein gradient for the whole body and a microgradient in individual buds (Chuong, 1991). It is possible there may be parallel macro and micro RA gradients in developing skin.

In other organs, alteration of axes in pattern formation also have been observed. In the developing nervous system of *Xenopus laevis*, RA causes an anteroposterior transformation (Durstson et al., 1989; Ruiz i Altaba and Jessel, 1991). In mice, vertebrae can be converted to more anterior or posterior structures by RA (Kessel et al., 1990) and in the marbled balloon frog RA causes homeotic conversion of tail to legs (Mohanty-Hejmadi et al., 1992). The floor plate and notochord can duplicate columns of motor neurons (Yamada et al., 1991). In the limb bud, a RA bead implanted to the anterior limb bud mimics the zone of polarizing activity (ZPA; Tickle et al., 1975) in causing mirror-image duplication of the A-P axis of limb digits (Eichele et al., 1985). Rather than reversing or duplicating the axis, our report is the first example of turning the orientation of the axis.

Alteration of Hox and N-CAM

What are the down-stream molecular events following the effect of RA that will eventually lead to the disorientation of feather buds? Hox genes are among the top candidates because they have been shown to be involved in the specification of body axis and limb axis (Cho et al., 1991; Ruiz i Altaba and Melton, 1989; Yokouchi et al., 1991; Nohno et al., 1991; Izpisua-Belmonte et al., 1991). Alteration of Hox genes by perturbing gene expression or by RA treatment have been shown to cause A-P transformation along the neural tube (Durstson et al., 1989; Ruiz i Altaba and Jessel, 1991). Microinjection of antibody to XIHBox 1 into one-cell *Xenopus* embryos causes posterior transformation of the tadpole midbrain (Wright et al., 1989). Transgenic mice over-expressing Hox 1.1 under the control of the beta-actin promoter causes posteriorization of cervical spine (Kessel et al., 1990). On the other hand, application of RA changes the Hox distribution in correlation with the altered axial patterns. In the limb bud, when the ZPA or a RA bead causes the duplication of digits, and the coordinated Hox 4 expression pattern in normal limb bud also becomes mirror-image duplicated (Izpisua-Belmonte et al., 1991; Nohno et al. 1991). When RA causes anterior or posterior transformation of vertebrae, the Hox distribution patterns (or Hox codes) change concomitantly with the specified vertebrae phenotype (Kessel and Gruss, 1991).

In the developing skin, we found homeobox protein gradients in developing feather buds (Chuong et al., 1990). It is indeed this background study that prompted us to search for the significance of homeoprotein expression in skin appendages. We therefore examined the expression of chicken proteins which cross-react with XIHbox 1 antibodies in RA-treated explants. The expression patterns of cross-reacting proteins with antibodies to the XIHbox 1 and Hox

5.2 homeoproteins in skin cultures were altered by treatment with RA. In these cultures, the homeoproteins lost their A-P gradient and exhibited a much more diffuse pattern (Fig. 7D), resembling the Hox expression pattern found in the scale (our unpublished data). We are currently mapping the complete expression pattern of a Hox complex in skin appendages from different locations of the body surface (Chuong, 1991) which will give more background information on the RA re-specification of skin appendage phenotypes and Hox codes. Early investigations suggested that the signal(s) encoding skin appendage phenotype were stored in the mesenchyme (reviewed in Sengel, 1976). We are currently testing the independent effects of RA on the mesenchyme and ectoderm through recombination experiments. Current data of Hox expression in RA-treated explants did not differentiate between the following possibilities: RA alters Hox expression which then alters skin appendage phenotype, or RA alters skin appendage phenotype which forced an altered Hox expression pattern, or RA exerts its effects upon skin appendage morphogenesis and Hox expression independently. Further proof on the causal relationship between homeobox expression and skin appendage phenotypes awaits a direct test by ectopically expressing homeobox genes in developing skin.

N-CAM is enriched in the anterior feather buds (Chuong and Edelman, 1985) and is involved in dermal condensations (Chuong et al., 1991; Jiang and Chuong, 1992). Our time course study (Fig. 5) showed that dermal condensations close to the RA bead appear to "dissolve" over a 4 day period. The buds with altered orientation appear to slow down during the dermal condensation stage (Fig. 5C). An examination of buds treated with RA in the media showed that the distribution of N-CAM became diffusive and enriched in the central and basal portion of the skin appendage (Fig. 7A, B). This suggests that RA can modulate the formation of dermal condensation, and this modulation may be mediated by adhesion molecules such as N-CAM.

The effects of RA on skin appendages would be most clear if we have specific molecular markers for them. While both alpha and beta keratins are present in feathers and scales, they each have unique spatio-temporal expression patterns (O'Guin et al., 1982). During the course of our explant cultures, both normal and RA-treated buds express alpha keratin but not beta keratin. The beta keratin would be expressed in more mature skin appendages. Further experiments with chorio-allantoic membrane culture or transplantation of these RA-treated skins to a host embryo will help us to determine what is the ultimate fate of these scale-like structures.

Other molecular changes to be considered are RA receptors (Giguere et al., 1987) and RA-binding proteins (Maden et al., 1989). In RA-induced glandular metaplasia of mouse vibrissae, there is an increased expression of beta RAR (Viallet et al., 1991). RARs are transcription factors that can influence subsequent gene expression. The signal transduction pathway for axis determination as well as an alteration in differentiation markers awaits further studies.

Although the retinoid species in developing skin remains to be determined, our preliminary data, based on HPLC analyses, suggest that there are dynamic qualitative and quantitative changes in retinoid expression patterns during development. These data which suggest the involvement of

retinoids in skin appendage morphogenesis will have to be verified by mass spectrometry. The observed effects of exogenous retinoids are retinoid species specific. We did not observe an effect of retinol either on beads or in the media, unless an extremely high concentration (eg., 10 μ M) was used which caused a complete inhibition of skin appendages (not shown). Retinal had a moderate suppressive effect, but we never observed the striking axial alteration effect or transformation to scale-like structures. The effect of RA beads is also concentration-dependent and this is nicely shown by the linear relationship between the areas of the inhibitory zone and the concentration of RA which was used to soak the anion exchange beads (Fig. 6). Due to the instability and photosensitivity of retinoids, there has been a discrepancy between the concentration and biological activity of RA. The linear relationship between the zone of inhibited feather bud formation to RA concentration may provide a straightforward bioassay to standardize the biological unit of morphogenetic activity of retinoids.

In the skin, both beta and gamma RA receptors have been reported to be present (Dolle et al., 1990; Zelent et al., 1989; Viallet et al., 1991). All these data strongly suggest that retinoids are involved in pattern formation. Although some work in limb development suggested that RA may not be the morphogen that directly alters the digit pattern (Noji et al., 1991; Wanek et al., 1991), the examples described in this discussion argue for a modulatory role, either physiological or non-physiological, played by RA on the fundamental mechanism of axis determination in body axis, limb axis and skin appendage axis.

Our overall hypothesis is that, as in the limb, the feather contains a zone of polarizing activity which is required to establish the feather axis. This signal is potentiated by the expression pattern of the Hox genes, which determines the orientation and phenotypes of vertebrate skin appendages as is the case for appendage phenotype determination in *Drosophila* (Gehring, 1985). RA has been shown to alter Hox expression pattern in a concentration- and time-dependent manner (Simeone et al., 1990). We surmise that treatment of skin appendages at the early forming stage "resets" the Hox codes, thus altering the orientation or phenotype of that skin appendage. Further study is required to demonstrate the direct role of Hox genes and to analyze the signal transduction pathway of phenotype determination of skin appendages.

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