Retinoids reprogramme pre-bud mesenchyme to give changes in limb pattern

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Summary

Retinoic acid was locally applied to presumptive limb regions of chick embryos to find out the earliest time at which the limb pattern can be reprogrammed. When beads soaked in retinoic acid were placed in the appropriate positions in embryos at stage 10 or older, duplicated or reduced leg patterns resulted. To pinpoint the time at which the cells in the limb rudiment respond to the retinoid, beads were removed at various times and the lengths of exposure required to reprogramme limb development found. The early limb rudiments require longer exposures to give duplications than late rudiments. The effective treatment periods last at least until stage 17 when the limb bud and apical ectodermal ridge develop. In contrast, the length of exposure to reduce the limb is constant at early stages. Retinoids first start acting to produce duplicated structures between stages 10 and 13. Therefore, retinoids appear to begin to reprogramme the cells as soon as they are determined to give rise to a limb.

Key words: limb development, retinoids, pattern formation, chick embryo.

Introduction

Experimental manipulations of developing chick limbs can bring about changes in the pattern of cellular differentiation across the anteroposterior axis (the axis running across the limb, for example, in the human hand from thumb to little finger). When tissue from the posterior margin of a chick wing bud (the polarizing region) is grafted to the anterior margin of another bud, duplicated patterns result (Saunders & Gasseling, 1968; Tickle, Summerbell & Wolpert, 1975). For example, six digits may develop instead of three; an additional set of digits 4 3 2 develops in mirror-image symmetry with the normal set 2 3 4. Local application of all-trans-retinoic acid to the anterior margin of chick wing buds has been found to mimic the signalling of polarizing region grafts (Tickle, Alberts, Wolpert & Lee, 1982; Summerbell, 1983). With appropriate concentrations of the retinoid, mirror-image symmetrical wing patterns develop (Tickle, Lee & Eichele, 1985).

The changes in the pattern of cellular differentiation just described result from manipulations when the bud is already formed. The early bud consists of a population of apparently homogeneous mesenchyme cells encased in ectoderm which has a thickened rim, the apical ectodermal ridge. However, the cells that will form the limb appear to be set aside much earlier in development. Tissues from the appropriate location along the body axis from chick embryos as early as stage 9, when neither bud nor thickened epithelium are present, will develop into leg structures when grafted to the coelomic cavity (Rudnick, 1945).

It is possible that the signals that order the pattern of cellular differentiation across the anteroposterior (a–p) axis operate before the definitive bud develops. Cells with detectable polarizing activity have been found in regions that will give rise to legs in embryos as early as stage 10 (Hornbruch & Wolpert, 1986). Alternatively, signalling may be required throughout limb development and may be linked to the pattern-forming process along the proximodistal (p–d) axis (running from the shoulder to the tips of the digits). The laying down of pattern along this axis is coordinated with bud outgrowth and depends on the apical ectodermal ridge (Saunders, 1948; Summerbell, 1974). To gain insight into this problem, we have
investigated whether retinoic acid can affect the pattern of cellular differentiation when applied to presumptive limb tissue before buds have developed.

Retinoids were applied to leg rudiments because we feared that application to the wing region of early embryos, near the developing heart, might cause embryo death (Jelinek & Kistler, 1981). We have found that a duplicated leg can result when embryos as early as stage 10 (10-somite stage) are treated with retinoic acid. The length of exposure to retinoic acid required to cause duplication in a limb skeleton is similar at stage 14 (22-somite stage) to the exposure required at a bud stage (20) (Eichele, Tickle & Alberts, 1985). However, embryos treated at stages 10 and 13 require longer exposures in order to induce duplicated structures in the leg. These results are discussed in relation to the timing of limb rudiment determination and the mechanism by which retinoids change limb pattern and its relation to normal polarizing activity.

Methods

Embryos

Fertilized hen eggs (Needle Farm, Herts) were incubated at 38°C. The eggs were windowed on days 2 and 3 and the embryos were staged according to Hamburger & Hamilton (1951).

Application of retinoids

AG1-X2 beads (in formate form: Biorad) were used to apply all-trans-retinoic acid (Sigma batch No. 104F.0135) to the leg buds or to regions of the flank where leg buds will develop. For stage-20 embryos, beads of 200 µm diameter were used; for earlier embryos the beads were 100 µm in diameter. The beads were soaked in solutions of all-trans-retinoic acid dissolved in DMSO, rinsed in tissue culture medium (see Tickle et al. 1985 for details of the procedure) and then implanted or positioned in the appropriate location in the embryo. In a short series of experiments, beads were soaked in solutions of a relatively stable analogue, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (TTNPB, Ro: 137410, a gift from Hoffman-LaRoche, Switzerland). As controls, beads were taken through the same procedure but were soaked in DMSO only.

Implantation of beads to leg buds of stage-20 embryos

The extraembryonic membranes were removed and a slit was made between the apical ectodermal ridge (AER) and the anterior margin or the apex of the right leg bud. The bead was placed between the free AER and the rest of the bud such that the bead was held in place. The procedure is described in full by Tickle et al. (1985).

Implantation of beads to leg regions of stage-14 to -16 embryos

The vitelline membrane was removed and the bead was placed in a small hole made in the right flank using a tungsten wire needle. Depending on the stage of the embryo, the hole was made in either somites 23–30 or in a position in the unsegmented lateral plate where these somites will shortly form. The leg bud is first recognized as a swelling in the flank opposite somites 26–32 at stage 17. It should be noted that in the bead removal experiments below, beads were placed on the vitelline membrane over stage-14 embryos.

Application of retinoids to stage 8–13 embryos

The bead was placed on the vitelline membrane over the right lateral plate in a position where the leg bud will later form. The positioning of the bead was determined by a series of preliminary experiments in which beads were placed in various positions along the lateral plate. The positions were recorded as the distance from the tail in camera-lucida drawings. In a few experiments, the bead was placed in the region where the wing will develop.

Effective length of exposure to retinoids

To find the length of exposure to retinoic acid required to affect the development of the leg pattern, beads were removed at various intervals after being placed on the vitelline membrane over the presumptive leg regions in stage-10 to -14 embryos. In control embryos, the beads were not removed.

In all experiments, following application of the retinoid, the eggs were resealed and incubated until the embryos were 10 days old.

Observation of the embryos following bead implantation

Following positioning of the bead in early embryos, a camera-lucida drawing was made before the egg was resealed and returned to the incubator. The next day, the position of the bead in relation to the early limb bud was noted.

Staining the embryos to show the pattern of cartilage differentiation

On the 10th day of incubation, the embryos were fixed in 5% TCA, stained in 0-1% Alcian green in 70% acid alcohol, dehydrated in ethanol and then cleared in methyl salicylate.

Scanning electron microscopy

In a set of experiments to investigate the effect of retinoic acid on bud formation, 100 µm beads soaked in 5 mg ml⁻¹ retinoic acid were placed on the vitelline membrane over the leg region of embryos staged 10–13. The embryos were fixed 24–48 h later in half-strength Karnovsky fixative (Karnovsky, 1965) for 24 h at 4°C. They were washed in 0.1 M cacodylate buffer, placed in osmium tetroxide dilute 1:1 with 0.2 M cacodylate buffer for 1 h, rinsed in 0.1 M cacodylate buffer and then dehydrated in ethanol. The ethanol was replaced with amyl acetate and the embryos
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Table 1. The skeletal patterns that develop following implantation of beads soaked in all-trans-retinoic acid beneath the apical ridge of leg buds of stage-20 embryos

<table>
<thead>
<tr>
<th>Concentration of retinoic acid in which beads were soaked (mg ml⁻¹)</th>
<th>Bead implanted anteriorly</th>
<th>Bead implanted at apex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total number</td>
<td>% duplicated digit pattern</td>
</tr>
<tr>
<td>0.1</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>86</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>67</td>
</tr>
</tbody>
</table>

were critical-point dried. The early leg buds were studied using a Jeol JSM-35 scanning electron microscope.

Results

Local application of retinoic acid to leg buds

Beads soaked in retinoic acid were implanted to the leg buds of stage-20 embryos. These experiments were done to provide data with which to compare the effects of retinoids applied before the leg bud has developed. Both the dose of retinoid and the position of application were varied. The results are summarized in Table 1.

When 200 μm beads soaked in 0.1 mg ml⁻¹ retinoic acid were implanted at the anterior margin of the leg bud, leg patterns were obtained with additional digits in mirror-image symmetry to the normal set. The pattern of digits in the majority of cases was III III II III IV or III III II III IV (the normal pattern is I II III IV, anterior to posterior, see Fig. 2A). When beads soaked in higher concentrations were implanted anteriorly, the number of digits progressively decreased. This was due to the loss of anterior digits either in duplicated patterns (IV III III IV, III III IV and IV III IV) or in reductions (II III IV and III IV). With the highest concentration of retinoic acid used, a duplicated fibula was often found in the foreleg and the tibia was missing. Local application of retinoic acid to the apex of the leg bud led to duplicated patterns at low doses (e.g. IV IV and II II) and complete truncations of the leg at higher doses.

Local application of retinoids to presumptive leg regions

Small 100 μm beads soaked in retinoic acid were implanted to the somites or the unsegmented lateral plate, or were placed on the vitelline membrane, in, or anterior to, the presumptive leg regions of embryos ranging from stages 8–16. For the earlier stages, a series of pilot experiments showed that the bead must be placed on the membrane 1 mm from the tail to end up in the region of the leg bud. The aim of this series of experiments was to find the earliest time at which beads soaked in the retinoid could lead to the development of duplicated leg structures. The results for beads soaked in 5 mg ml⁻¹ retinoic acid are shown in Fig. 1.

When beads soaked in 5 mg ml⁻¹ retinoic acid were placed on stage-8/9 embryos, the embryos usually died. The legs of surviving embryos were normal. However, in one case (out of two) when beads soaked in the relatively stable retinoid TTNPB (0.005 mg ml⁻¹) were placed on stage-9 embryos, a leg with a single additional digit (I I III IV) resulted. When beads soaked in 5 mg ml⁻¹ retinoic acid were placed in the appropriate position in embryos at stage 10, duplicated leg patterns developed (see Fig. 2). However, many of the legs of embryos treated at this stage were normal or had missing structures. Some had no leg at all. At stages 11–14, beads soaked in the same concentration of retinoid were more successful in producing duplications and the percentage of normal or reduced/missing patterns fell (Fig. 1). Retinoid treatment at stages 10–14 could also cause the loss of the tail and/or vertebrae. Apart from treatment at
stage 8/9, the survival rate of treated embryos was more than 80%.

Although the incidence of leg duplications generally increased with stage at treatment, the quality of the duplications did not vary. Legs with an additional digit IV (the most posterior digit) could develop after application of a bead soaked in 5 mg ml$^{-1}$ at all stages and the maximum number of digits usually attained (6) was also the same irrespective of stage at treatment (see Fig. 4). Fig. 3 shows examples of duplications following retinoid application to embryos between stages 13 and 20.

It should be noted that the greatest duplicating effect on the pattern was found in the digits. The foreleg could be duplicated but no more than three elements (fibula, tibia, fibula) (Fig. 3B) instead of the
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normal two (tibia, fibula) were ever formed. The foreleg elements could both be fibulae. However, frequently only one foreleg element, the fibula, was present (Fig. 2A). In no case was the femur duplicated even when the retinoid was applied at stage 10.

To determine whether the mixture of duplications and reduced leg patterns obtained at the earliest stages (10–13), was due to the position of the bead relative to the limb field, the pattern of digits was compared with the distance from the tail at which the bead was placed. This analysis revealed no consistent trend. This was probably due to the variability of bead displacement following positioning on the membrane over the leg region. However, when the position of the bead in relation to the bud that developed 1–2 days later was compared with the digit pattern, a position-dependent effect was revealed (see Fig. 4A–C which were prepared from the data in Fig. 1). When beads ended up at the anterior part of the early bud, duplicated leg patterns could develop, whereas when beads ended up in more posterior positions, usually the legs had structures missing.

Fig. 3. Duplicated leg patterns resulting from treating stage-13 to -20 embryos. (A) Treated at stage 13; bead soaked in 5 mg ml⁻¹ retinoic acid. Digit pattern: II I II III IV. Bead at anterior of bud at stage 17. (B) Treated at stage 14; bead soaked in 5 mg ml⁻¹ retinoic acid. Digit pattern: IV III II III IV; two fibulae and one tibia in foreleg. Bead at anterior of bud at stage 17. (C) Treated at stage 20; bead soaked in 1 mg ml⁻¹ retinoic acid implanted beneath the apical ridge at the anterior margin of leg bud. Digit pattern: IV III III IV.

It should be noted that when the bead releasing retinoid ended up well anterior to the leg bud, the development of the flank was normal. When beads soaked in retinoic acid were placed on the membrane even more anteriorly, over the presumptive wing region, the pattern of the wing could be affected at least as early as stage 10.

In addition to the position-dependent effects of retinoid application, there was also a dose response (Table 2A,B). When beads soaked in 1 mg ml⁻¹ rather than 5 mg ml⁻¹ retinoic acid were placed in the presumptive leg region, the frequency and extent of duplications were decreased. Reduced leg skeletons rarely resulted with beads soaked in 1 mg ml⁻¹ retinoic acid. Control beads, soaked in DMSO alone, did not affect the pattern of the legs.

The earliest time at which retinoids affect leg development

The data in Fig. 1 and Fig. 4A show that duplicated legs can develop when beads soaked in retinoic acid are placed on the vitelline membrane above the presumptive leg regions in stage-10 embryos. However, it is possible that the retinoid does not exert its effect on leg development until some later stage. To investigate this question, a series of experiments was carried out in which beads were placed on the membrane of stage-10 embryos and then removed at later times. The length of exposure to retinoic acid required to bring about pattern changes after application at stage 10 was then compared with the exposure required at stages 13 and 14 (N.B. the results of treatment of stage-14 embryos were very similar when beads were placed on the membrane or were implanted to the embryos, these data are not
Fig. 4. Beads soaked in 5 mg ml\(^{-1}\) retinoic acid were placed in presumptive leg regions of early embryos. Digit patterns in relation to the position of the bead at bud formation at stage 17. This figure was prepared from the data in Fig. 1. N.B. In this figure Arabic numerals rather than the usual Roman are used to denote the leg digits. (A) Stage-10 embryos; (B) stage-13 embryos; (C) stage-14 embryos. □ 10 cases, otherwise the side length of the square indicates the number of cases: the smallest square represents one case. * Somites.

Table 2. The effects of beads soaked in all-trans-retinoic acid applied to presumptive leg regions of early embryos on the skeletal pattern of the legs that developed

<table>
<thead>
<tr>
<th>Concentration of retinoic acid in which beads were soaked (mg ml(^{-1}))</th>
<th>Anterior to bud – centre bud*</th>
<th>Centre bud – posterior to bud*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage treated</td>
<td>total survivors</td>
</tr>
<tr>
<td>0</td>
<td>10–13</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>10–13</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>11–12</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration of retinoic acid in which beads were soaked (mg ml(^{-1}))</th>
<th>Implantation to somites 23–29</th>
<th>Implantation to somites 29–30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage treated</td>
<td>total survivors</td>
</tr>
<tr>
<td>1</td>
<td>14–16</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>14–16</td>
<td>45</td>
</tr>
<tr>
<td>10</td>
<td>14–16</td>
<td>5</td>
</tr>
</tbody>
</table>

Leg bud develops between somites 26–32.

* Position of bead relative to leg bud at stage 17.
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Fig. 5. Beads soaked in 5 mg ml\(^{-1}\) retinoic acid were placed on the vitelline membrane over the presumptive leg regions of early embryos. At a series of later times the beads were removed. The effects of different lengths of exposure to the retinoid on the pattern of the legs that developed are shown. D, normal; M, some reduced, some normal; E, some duplicated, some normal; B, some limbs duplicated, some reduced and some normal. (A) Stage-10 embryos, (B) stage-13 embryos; (C) stage-14 embryos.

Fig. 5 compares the resulting leg patterns when beads were removed from the vitelline membrane of embryos treated at stages 10, 13 and 14. Short exposures of 6 h resulted in normal leg skeletons. Duplicated leg patterns were obtained following treatment at stage 10 for 29 h, whereas at stage 13 an exposure of 24 h led to duplications and at stage 14 an exposure of 18 h was required. Therefore, the retinoid-releasing bead is in place until stage 17, stage 17/18 and stage 18 respectively. In contrast to the differences in length of exposure required for duplications, an 18 h exposure to retinoic acid could lead to leg reductions or truncations when embryos were treated at stage 10 or 13.

An exposure to retinoic acid that ends just as the bud is forming can be effective in altering the pattern of leg structures. Indeed, the early application of retinoic acid results in changes in the form of the bud that develops. Fig. 6 shows leg buds that developed following retinoic acid application at stages 10–13. As early as stage 17/18, the shape of the treated leg bud was altered. The buds were either wider in the a–p axis with the AER extending further anteriorly than in the control left bud or they were narrow in the a–p axis with a short AER. These buds would probably go on to form duplicated or reduced limbs, respectively.

Discussion

The earliest stage of development at which retinoic acid can be locally applied and lead to changes in the pattern of the leg is stage 10 when no bud has yet formed. The response to the retinoid is dose and
Fig. 6. Leg buds that developed following treatment of the limb rudiment (on the right side of the embryo) at earlier stages with retinoic acid. (A) Treated stage 10. Drawing of tail end of embryo (photograph of specimen on right). Right bud at stage 17/18 is broad across the anteroposterior axis. Outgrowth is extended anteriorly. Compare with left bud. The anteroposterior extent of the right and left buds and their apical ridges are arrowed. (B) Treated stage 12. SEM of tail end of embryo viewed from ventral surface. Right bud at stage 23 is narrow across the anteroposterior axis and the apical ectodermal ridge is short.

Position dependent as it is for leg-bud stages. Progressively shorter exposures to retinoic acid are required by stage-10, -13 and -14 embryos to duplicate leg pattern. They end at stage 17/18 when the bud and apical ridge develop.

Stage, position and dose effects
Local application of retinoic acid to embryos of a wide range of developmental stages, including both pre-bud and leg-bud stages leads to the development of duplicated leg structures. The incidence of duplications obtained by treatment of pre-leg-bud mesenchyme increases with developmental stage to around 50% for stage-13 to -14 embryos. The more effective treatment at these stages may reflect the reduction in the length of exposure required to duplicate pattern. This increases the likelihood that the bead remains in the proper position long enough for duplications to result. Especially at the earliest stages, the bead may translocate on the membrane. 100% duplications are formed when beads are implanted in contact with the AER at the anterior margin of stage-20 leg buds. This suggests that interaction of the AER and mesenchyme may be required for changes in the pattern (see later).

The patterns of duplications appear to be independent of developmental stage. An additional digit IV is just as likely to develop in a duplicated pattern following treatment at stage 10 as at stage 14. Therefore, the response to retinoids of the limb rudiment at different stages is equivalent.

The effect of retinoids is clearly position dependent when applied to limb buds. The duplications occur when the retinoid source is at the anterior of the bud. When the bead is at the apex or at the posterior of the bud, the limb can be reduced (Tickle et al. 1985; Lee & Tickle, 1985). The patterns of legs obtained following treatment at pre-bud stages similarly appear to correlate with the position of the bead relative to the bud that forms. Duplications mainly occur when the bead ends up anteriorly, whereas a mixture of duplications and reductions occur when the bead ends up near the centre of the bud. However, embryos treated at stages 10–13 are particularly susceptible to limb reduction. This may relate to the small size of the limb rudiments at these early stages (Chaube, 1959).

The extent of the changes in pattern depends on the dose of retinoid applied at both pre-bud and leg-bud stages (for wing-bud stages, see also Tickle et al. 1985). However, we cannot directly compare the doses because the beads used for releasing the retinoid into presumptive limb tissue are smaller than those used to treat the limb bud. In addition, the beads are directly implanted into the leg tissue at bud stages rather than being placed nearby or on the membranes.

Exposure time to retinoic acid required to register pattern changes
When placed on stage-10 embryos, beads soaked in retinoic acid left in position for 29 h produce duplicated leg patterns; whereas a 24 h exposure is sufficient for stage-13 embryos and 18 h for stage-14 embryos. The time for stage-14 embryos is the same as that required to give full duplications in stage-20 wing buds (Eichele et al. 1985). The longer exposure to retinoic acid that leads to duplications after treatment at stage 10 compared with stage 14 could suggest that the limb rudiment may only respond to retinoic acid during the last 18 h of treatment. In this case, the effective exposure would begin between 11–15 h after stage 10 at stage 13(+). However, stage-13 embryos
require more than an 18 h exposure to duplicate the pattern. This argues against the cells first responding at stage 13 and instead suggests a changing time requirement to register pattern duplications during early development. The duplicating effect of TTNPB when applied at stage 9, rather earlier than retinoic acid, can be explained by the longer half life of this retinoid in chick embryonic tissue (Eichele et al. 1985).

In contrast to the changing exposures required for pattern duplications at stages 10-14, the length of time required for limb reductions is 18 h for stages 10 and 13. The different exposures required for these two effects are difficult to reconcile with the assumption that the underlying mechanism of retinoid action is the same irrespective of position: the different effects on limb pattern being due to interactions with the signal from the native polarizing region (see Lee & Tickle, 1985).

**Retinoid effects on bud outgrowth**

When a bead soaked in retinoic acid is positioned above the leg region of stage-10 to -14 embryos, the bead must remain in place until at least stage 17 for a duplicated leg skeleton to result. Since the leg bud first grows out from the flank at stage 17, the retinoid may be required at this stage to affect the development of the apical ectodermal ridge. However, the amount of retinoid being released from the bead must be considerably reduced by this stage (Eichele et al. 1984, 1985). Even as the bud first grows out, the shape of the bud and extent of the AER are already changed compared with the contralateral control bud. Changes in the extent of the apical ridge that lead to the modification of bud shape can also be detected as early as 24 h later when retinoic acid is applied to limb buds (Lee & Tickle, 1985). Therefore, shape change occurs around 24 h after treatment, independent of the stage at which retinoid is applied. The changes in shape may be closely linked to the registering of the retinoid signal. This should be contrasted with the formation of additional digits that can occur at quite variable time intervals after retinoid treatment.

**The state of the limb cells when retinoids first begin to affect the future limb pattern**

Our data suggest that retinoids start to act on the limb rudiment between stages 10 and 13. This correlates with the stage (11) at which Pinot (1970) found that isolated prospective leg regions, plus adjacent axial mesenchyme, could first develop into leg structures. However, Rudnick (1945) found that leg structures could develop from appropriate regions as early as stage 9. In this case, Hensen's node must be included in the transplant. Since Hensen's node, at this stage, has polarizing activity (Hornbruch & Wolpert, 1986), interactions may take place within the transplanted tissue that lead to the subsequent determination of the limb region. Retinoids appear to be able to start to reprogramme the pattern as soon as the mesenchyme cells are determined. They appear unable to induce the formation of a new limb field.

**Mode of retinoid action**

Leg duplications can arise without wounding the tissue. Treatment of stage-10 to -13 embryos involved simply placing the bead on the vitelline membrane over the presumptive limb area. Therefore, retinoids do not appear to act in concert with growth stimulated at cut surfaces as suggested by Scadding & Maden (1986).

From experiments on limb buds, it has been found that implantation of beads soaked in retinoids leads to the establishment of a stable retinoid gradient (Tickle et al. 1985; Eichele et al. 1985). A graded distribution of retinoid is more effective in producing pattern changes than the same amount of retinoid distributed evenly (Eichele et al. 1985). One possibility is that the gradient of retinoid may directly modify pattern across the a-p axis of the limb. A second possibility is that only the high point of the gradient is effective and that this locally transforms adjacent mesenchyme into a new signalling region.

Morphogen gradients are believed to be involved in specifying the anteroposterior axis in Drosophila embryos. For example, a graded distribution of the product of a homeobox-containing gene has been found early in the development of the fertilized Drosophila egg (Macdonald & Struhl, 1986). However, there is the analogous problem about how such gradients are read. In this example, the important factor in specifying pattern may not be the early graded distribution but instead the subsequent localization of the gene product at the posterior of the Drosophila embryo.

The results of the experiments here do not distinguish between the two possible mechanisms for retinoid-induced changes in presumptive limb regions that lead to altered patterns in the legs. In the first mechanism, the effects of retinoic acid would have to bring about stable changes in the limb rudiment such that extra structures are formed several days later. In the second mechanism, retinoids would transform presumptive limb cells into a new polarizing region which then signals along with the native polarizing region.

**When does the polarizing region signal in limb development?**

In amphibian embryos, grafts of regions of the flank posterior to the limb rudiment, when placed anterior
to the rudiment can lead to the development of duplicated limbs (Slack, 1976, 1977a,b). Hornbruch & Wolpert (1986) have found polarizing activity as early as stage 10 in the leg region of chick embryos by grafting tissue to wing buds. It is also well established that posterior tissue from leg buds can cause duplications when grafted to anterior positions in both leg and wing buds (Summerbell & Tickle, 1977). Therefore, one interpretation is that the polarizing region begins signalling at the time that the limb rudiment is determined and continued signalling is required throughout subsequent limb development. A second possibility is that the polarizing activity detected by grafting experiments does not reflect signalling in normal limb development and the tissue only becomes active when transplanted.

In bud stages, the requirement for the polarizing region throughout limb development is suggested by the defective wings that develop after removing a slice from the posterior of the bud (Hinchliffe & Gumpel-Pinot, 1981) and following insertion of an impermeable barrier between anterior and posterior halves of the bud (Summerbell, 1979). On the other hand, removal of tissue from buds between stages 19 and 24, normally used for polarizing region grafts, does not affect limb development (Fallon & Crosby, 1975). Furthermore, only a short exposure to the signal from the grafted polarizing region is sufficient to bring about pattern changes (Smith, 1979, 1980).

The time when signals operate is relevant to the analysis of the mechanisms of signalling in normal limb development. For example, when are the genes transcribed that act in the pattern-forming process across the anteroposterior axis of the limb? These experiments with retinoids do not resolve this issue. However, retinoids can bring about pattern changes with defined time/dose relationships. Therefore, the application of retinoids will allow the analysis of events when a signal that affects anteroposterior pattern is registered.

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References


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