Retinoic acid and chick limb bud development

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Summary

The chick limb bud is a powerful experimental system in which to study pattern formation in vertebrate embryos. Exogenously applied retinoic acid, a vitamin A derivative, can bring about changes in pattern and, on several grounds, is a good candidate for an endogenous morphogen. As such, the local concentration of retinoic acid might provide cells with information about their position in relation to one axis of the limb. Alternatively, retinoic acid may be part of a more complex signalling system.

Homeobox genes are possible target genes for regulation by retinoic acid in the limb. In particular, one homeobox gene, *XlHbox 1* is expressed locally in the mesenchyme of vertebrate forelimbs and might code for an anterior position. When the pattern of the chick wing is changed by retinoic acid or by grafts of signalling tissue such that anterior cells now form posterior structures, the domain of *XlHbox 1* expression expands rather than contracts. The expansion of *XlHbox 1* expression correlates with shoulder girdle abnormalities.

Retinoic acid application leads to visible changes in bud shape and this allows dissection of the way in which patterning is co-ordinated with morphogenesis. Results of recombination experiments and studies of changes in the apical ridge and proliferation in the mesenchyme suggest the following scheme: retinoic acid is involved in specification of position of mesenchyme cells; this specification determines their local interaction with the ridge that controls ridge morphology; the thickened apical ridge permits local proliferation in the underlying mesenchyme.

The recent advances in molecular biology that permit analysis of the expression of various interesting genes in developing limbs hold out the promise that further investigation may soon allow a complete account of the patterning process in one part of the vertebrate embryo.

Key words: chick limb bud, retinoic acid, homeobox gene, morphogenesis.

Introduction

The development of the chick limb has provided a powerful system in which to study pattern formation in vertebrates. The early limb is a small bud of mesenchyme cells that gives rise during development to spatially defined patterns of differentiated cells. The concept that has been particularly influential in the analysis of this system is that of positional information (Wolpert, 1969, 1989). According to this view, cells are first informed of their position within the limb bud and acquire a positional value. The cells then interpret the positional information and differentiate according to their position.

Extensive investigations over a number of years have built up a picture of the cell interactions that inform cells of their position in the bud. More recently a significant advance was the finding that retinoic acid, a derivative of vitamin A, can reproducibly alter the pattern of differentiated cells along one axis of the chick limb (Tickle *et al.* 1982). Subsequent work has demonstrated that retinoic acid is present in developing

chick limbs (Thaller and Eichele, 1987) and this has firmly established retinoic acid as a good candidate for an endogenous signalling substance. However, retinoic acid must interact with other signals, for example those that inform cells of their position along the other two axes of the limb. In addition, patterning must be co-ordinated with morphogenesis, the process that shapes the developing limb and involves growth.

Over the last few years there has been rapid progress in identifying molecules that are expressed in developing limbs of vertebrates and that could play an important role in limb patterning and morphogenesis. Three main classes of molecules are of potential interest. One such class comprises the growth factors. It is now clear that growth factors can function as signalling molecules in early amphibian embryos (reviewed by Slack *et al.* 1989; J. C. Smith *et al.* 1989), and their distribution in mouse embryos suggests a role in epithelial–mesenchymal interactions (Heine *et al.* 1987; Lehnert and Akhurst, 1988). Two other classes of molecules with potential roles in patterning are expressed in developing limbs. Nuclear receptors for

retinoic acid could directly mediate the response to retinoic acid (Giguere *et al.* 1987; Petkovich *et al.* 1987), while homeobox genes could encode positional values.

Cell interactions in limb development

Three main sets of interactions have been identified in the developing chick limb bud (Fig. 1). One set of epithelial–mesenchymal interactions involves an interplay between the apical ectodermal ridge, the thickened epithelium at the tip of the limb bud and the underlying mesenchyme. The apical ridge is required for bud outgrowth (Saunders, 1948; Summerbell, 1974*a*) and maintains the progress zone, a region of undifferentiated cells at the tip of the limb. The length of time cells spend in the progress zone appears to inform them of their position along the proximo–distal axis of the limb (Summerbell *et al.* 1973).

A second set of epithelial–mesenchymal interactions involves the ectoderm encasing the sides of the bud. Rotation of the ectoderm affects the pattern of structures across the dorso–ventral axis of the limb (MacCabe *et al.* 1974). While ectoderm inhibits cartilage differentiation of limb mesenchyme cells in high density culture (Solursh, 1984; Gregg *et al.* 1989), denuding a limb bud of large areas of ectoderm had no detectable effect on the position in which cells differentiate into cartilage (Martin and Lewis, 1986).
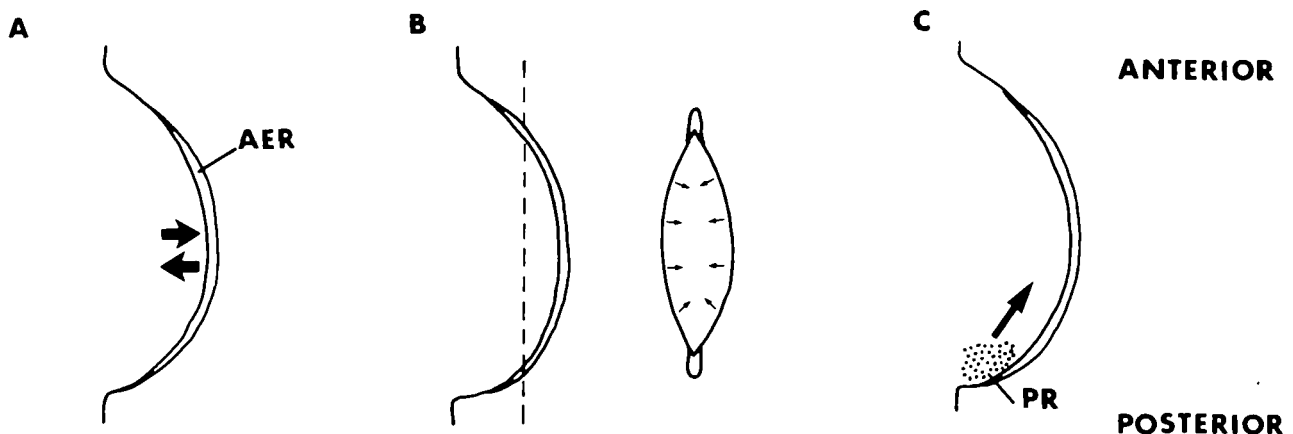
The third set of interactions involves the polarizing region, a small group of mesenchyme cells at the posterior margin of the limb bud (Saunders and Gasseling, 1968). A signal from the polarizing region acts on mesenchyme cells in the progress zone and determines the pattern of structures that develop across the antero–posterior (a–p) axis of the limb (Tickle *et al.* 1975; Summerbell, 1974*b*).

Signalling by retinoic acid in the chick limb bud

Grafts of the polarizing region to the anterior margin of the wing lead to mirror-image duplications of pattern (Tickle *et al.* 1975). A signal from the graft leads anterior cells to form posterior structures. In particular, the digits provide good markers for position along the a–p axis. Following a polarizing region graft, six digits develop instead of three and the duplicate set, 4 3 2 is formed in addition to the normal set 2 3 4 (Fig. 2A,B). When retinoic acid is locally applied to the anterior margin on small controlled-release carriers, mirror-image duplicated patterns are also produced (Fig. 2C,D).

The signalling induced by local application of retinoic acid and grafts of polarizing region tissue show striking similarities: the effects on pattern are dose-dependent and position-dependent, and the length of exposure to produce pattern changes is the same (reviewed by S. M. Smith *et al.* 1989). The addition of digits to the wing pattern is sequential. Wing patterns with an additional digit 2, additional digits 2 and 3 and finally with a complete set 2 3 4 can form. Each extra digit can be produced by either increasing retinoic acid concentration or by increasing the length of exposure to it (Tickle *et al.* 1985; Eichele *et al.* 1985). An exposure of about 18 h is sufficient to specify a complete set of additional digits with the appropriate dose of retinoic acid. Continuous application of retinoic acid is not required. Exposure to retinoic acid can affect pre-bud mesenchyme that is determined to give rise to the limb (Wilde *et al.* 1987) and formation of the additional digits occurs several days after the bead soaked in retinoic acid is removed. This quite clearly shows that the applied retinoid is acting on the patterning process and not on differentiation of cells into the tissues of the digits.

The graded distribution of a substance could convey positional information. Carriers soaked in retinoic acid

Fig. 1. Cell interactions in the chick wing bud. (A) Reciprocal epithelial–mesenchymal interactions between the apical ectodermal ridge (AER) and underlying undifferentiated mesenchyme. The apical ridge is required for bud outgrowth and maintains the progress zone at the tip of the limb. (B) Limb bud dissected along dashed line and end-on view of cut surface shown on the right. The arrows depict a postulated signal from the epithelium that controls the pattern of differentiation across the dorso–ventral axis of the limb. (C) Signal from the polarizing region (PR), a small group of mesenchyme cells at the posterior margin of the limb bud, which acts on mesenchyme cells at the tip of the limb bud and is involved in patterning across the a–p axis of the limb.
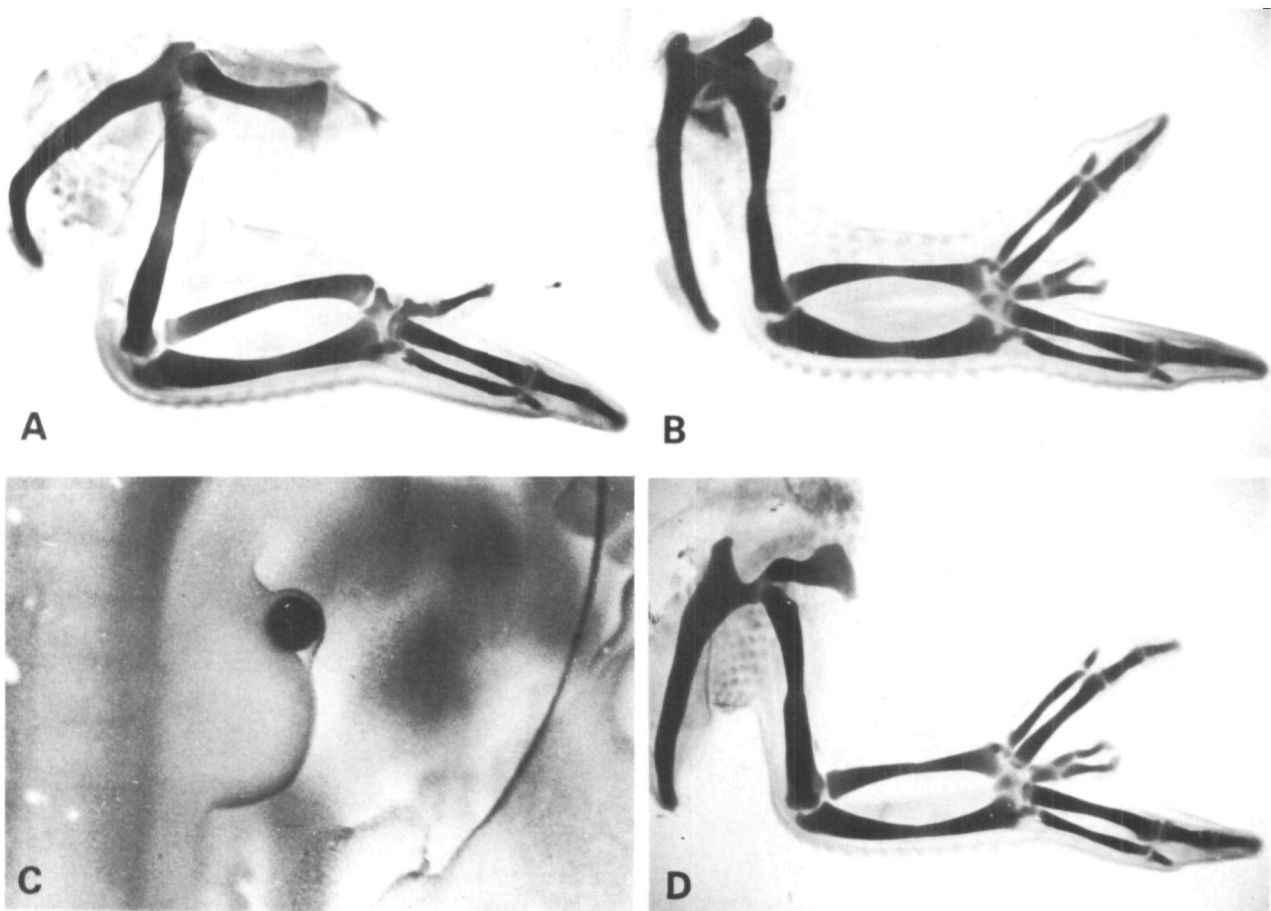
Fig. 2. (A) Normal pattern of cartilage elements in embryonic skeleton of chick wing shown in whole mount. The three digits are from anterior (top), **234**. (B) Wing skeleton with duplicated pattern of digits following graft of polarizing region tissue to anterior margin of an early bud. Reading from anterior (top), digit pattern is **432234**. (C) Bead that acts as a controlled release carrier implanted at anterior margin of early wing bud. The apical ectodermal ridge is cut away from the mesenchyme and forms a loop which keeps the bead in place. (D) Wing skeleton with duplicated pattern of digits following implantation of a bead soaked in $0.1\,\mathrm{mg\,ml^{-1}}$ retinoic acid as shown in (C). Digit pattern is **432234**.

release the retinoid so that a gradient of retinoic acid is established across the bud (Tickle *et al.* 1985). Cells at different distances from the carrier will be exposed to different concentrations of retinoic acid. Thaller and Eichele (1987) showed that retinoic acid is present in normal chick wings and is enriched in the posterior part of the bud where the polarizing region is located. In contrast, retinol from which retinoic acid can be generated is present in higher concentrations and is evenly distributed. One could therefore envisage a model in which signalling of the polarizing region involves the conversion of retinol into retinoic acid which diffuses across the bud; the local concentration of retinoic acid to which cells are exposed would specify position along the a–p axis. Thaller and Eichele (1988) showed that retinol applied to the posterior margin of the chick wing bud is converted into retinoic acid.

Very recently a second pathway of retinol metabolism in chick wing buds has been discovered that produces a metabolite that affects patterning. 3,4-didehydroretinoic acid is present in the wing and also induces additional digits when applied anteriorly

(Thaller and Eichele, 1990). It appears that the signalling system generated by vitamin A metabolism may be more complex than at first envisaged.

An important question is whether retinoic acid (or another retinoid) directly acts as a positional signal. Although a gradient of retinoic acid is more effective in specifying additional digits than the same amount distributed evenly across the bud (Eichele *et al.* 1985), there is no direct evidence that cells read the local concentration of retinoic acid to find their position. When gap junction antibodies are used to block cell–cell communication between polarizing region cells and responding anterior mesenchyme cells in mixed grafts, a small but significant reduction in the efficiency of respecification of host wings can be detected (Allen *et al.* 1990). The interaction between polarizing region and anterior mesenchyme cells presumably involves the passage of small regulatory molecules but its relationship to the production of retinoic acid is not known. One possibility is that retinoic acid treatment leads to the creation of a new polarizing region which then signals *via* gap junctions.
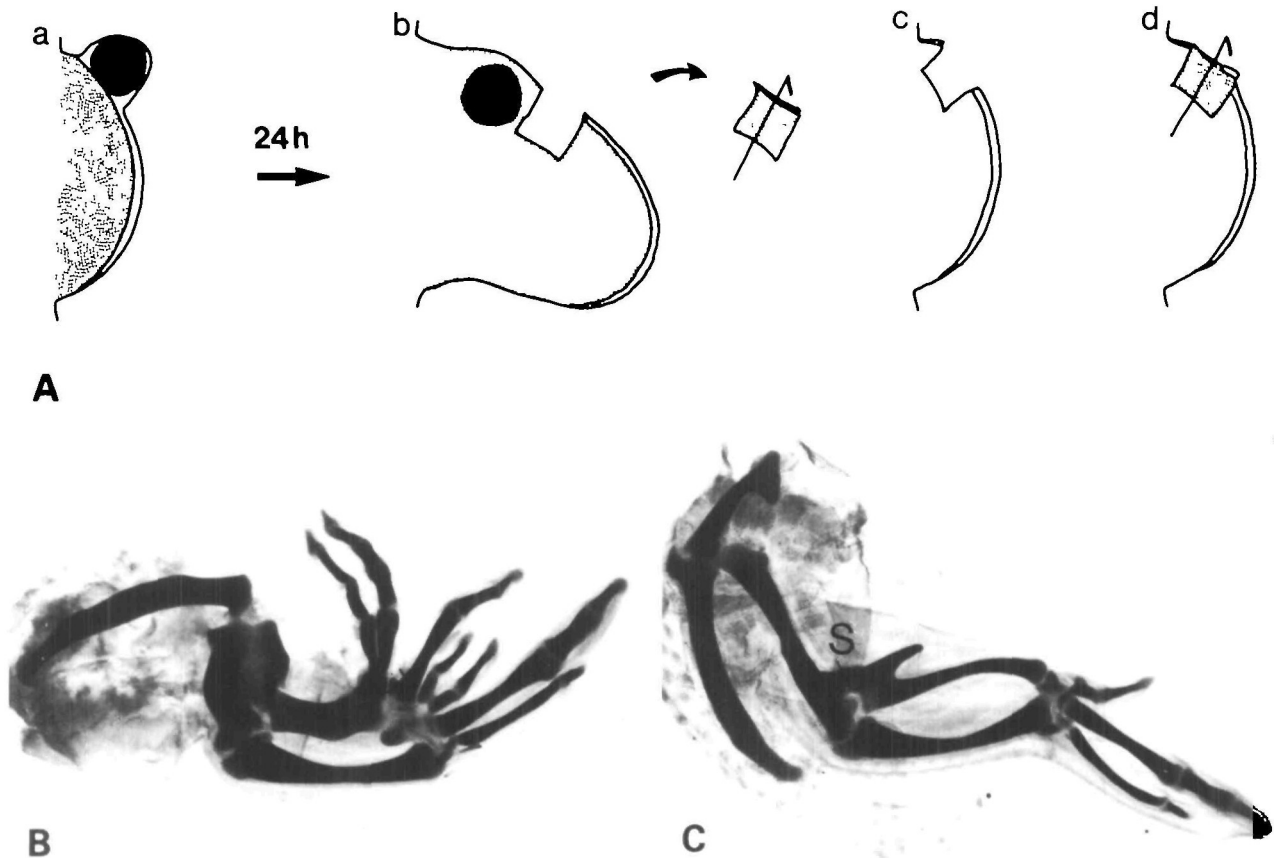
Fig. 3. (A) Schematic diagram showing an experiment to test whether cells exposed to retinoic acid in the limb bud acquire polarizing activity. a, Bead soaked in retinoic acid is implanted to the anterior margin of an early leg bud. b, 22–24 h later, leg tissue adjacent to the bead is cut out and speared with a platinum pin. c, Hole cut in anterior margin of an early wing bud. d, Leg tissue pinned into hole in wing bud. (B) Wing that developed following graft of tissue from a leg bud. Bead soaked in 0.1 mg ml^{-1} retinoic acid was implanted to anterior of leg bud and tissue assayed 24 h later. Two of the additional digits are toes, but there is also an additional wing, digits 3 and 2. (C) Wing that developed following graft of tissue from leg bud. Bead soaked in 10 mg ml^{-1} retinoic acid was implanted to leg bud and tissue assayed 8 h later. No additional digits developed. S is spur of cartilage formed by graft.

A further possibility is that retinoic acid by-passes, in some way, an earlier interaction between polarizing region and anterior mesenchyme cells. These considerations suggest that although retinoic acid has a central role in controlling pattern across the a–p axis, other signals may be involved.

Some preliminary data suggest that tissue in the limb bud does acquire polarizing activity when exposed to retinoic acid. When tissue adjacent to a bead soaked in retinoic acid is grafted to a second wing bud, additional digits develop in the host wing (see also Summerbell and Harvey, 1983). To exclude the possibility that the additional digits arise as a result of self-differentiation of the grafted tissue, beads soaked in retinoic acid were implanted to the anterior margin of either a wing bud or a leg bud. 22–24 h later, when irreversible specification of a mirror-image pattern has taken place, the tissue next to the bead was grafted to the anterior margin of a second limb bud (Fig. 3A). Tissue from a wing bud was grafted to a leg bud and tissue from a leg grafted to a wing. When leg bud tissue taken adjacent to a bead is

grafted to a wing bud, additional digits develop in the host wing bud (Fig. 3B). In the wing illustrated, two of the additional digits are clearly toes and must be derived from the graft. However, the other two additional digits are wing digits 3 and 2 and must have arisen from the host in response to a signal from the graft. When the grafted tissue is taken from the leg bud only 8 h after implantation of a bead, no additional digits form (Fig. 3C). Since the concentration of exogenously applied retinoic acid soon reaches a steady state in limb buds (Eichele et al. 1985), this result indicates that carry-over of retinoic acid in the grafted tissue probably does not account for the formation of additional host-type digits. The acquisition by anterior cells of signalling characteristics of polarizing region cells could be part of the respecification of anterior cells to form the most posterior part of a mirror-image pattern. These new polarizing region cells might, in principle, generate a distinct signal that passes through gap junctions or it may be that an autocatalytic process could operate so that a retinoid signal is produced. So
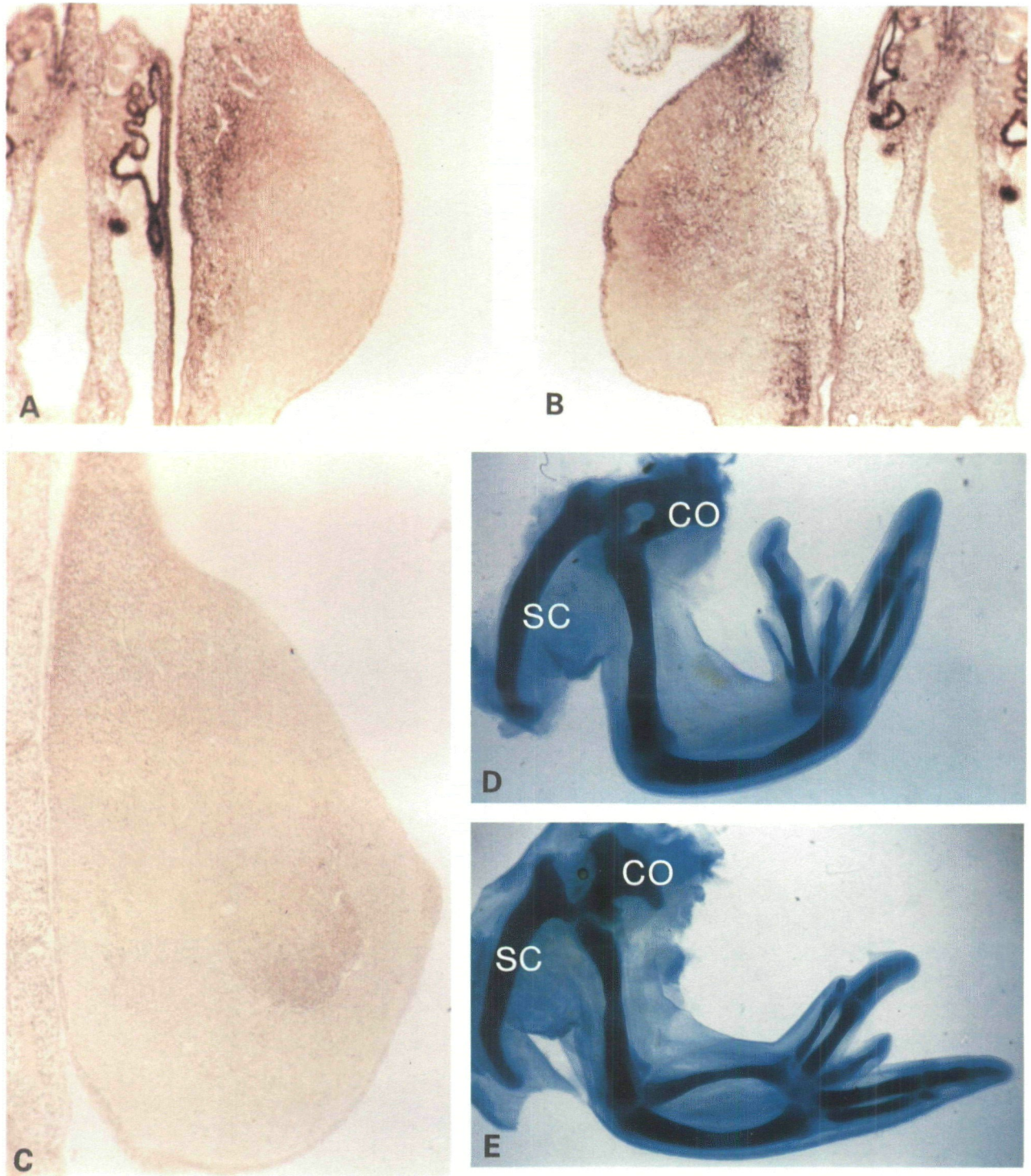
Fig. 4. Expression of a homeobox gene in chick wing buds and the shoulder girdle abnormalities correlated with changes in gene expression following retinoic acid treatment. (A–C) Frontal sections through chick wing buds stained with anti-*XlHbox 1* antibodies. Anterior is at the top. Photographs kindly provided by Drs Oliver and De Robertis. (A) Normal wing bud showing *XlHbox 1* expression anteriorly and proximally in the mesenchyme. (B) Chick wing bud 18 h after implantation of a bead soaked in $1\,mg\,ml^{-1}$ retinoic acid at the apex of a very early bud (stage 18). *XlHbox 1* expression in the mesoderm is greatly expanded. Note that the ectoderm is also thickened and shows increased staining over the anterior part of the bud. (C) Anterior mesenchyme transplanted into the posterior of the wing bud. *XlHbox 1* is still expressed in the grafted cells 18 h after transplantation. (D) Wing that developed after implantation of bead soaked in $0.1\,mg\,ml^{-1}$ retinoic acid at stage 18. Coracoid (CO) is short and appears to be duplicated. CO, coracoid, SC, scapula. (E) Wing after the same treatment as in (D). Coracoid is fused with the scapula. Elements on either side of the joint appear symmetrical. The head of the coracoid resembles that of the scapula.

far, attempts to convert anterior limb bud cells into polarizing region cells in culture have been unsuccessful.

Genes with a potential role in patterning

Nuclear receptors for retinoic acid enable cells to detect and respond to retinoic acid. This class of molecules is turning out to be quite complex, with two apparently separate families identified, each with several types of receptor, some of which exist in different isoforms (reviewed by Ragsdale and Brockes, 1990; Mangelsdorf *et al.* 1990). In addition to the nuclear receptors, cellular retinoic acid binding proteins have been identified (Chytil and Ong, 1984) which could modulate the amount of retinoic acid available to interact with the receptors. Retinoic acid receptors and cellular binding proteins are expressed in developing vertebrate limbs in complex temporal and spatial patterns (Dollé *et al.* 1989a, Maden *et al.* 1988) and it will be important to understand the roles of the different receptors and binding proteins.

The nuclear receptors provide the molecular mechanism by which retinoic acid can control gene expression. Possible candidates for genes regulated by retinoic acid are homeobox genes. In teratocarcinoma cells, changes in expression of such genes can occur rapidly following retinoid treatment (La Rosa and Gudas, 1988). When retinoic acid is applied to developing chick limb buds, anterior cells form posterior structures and therefore the expression of genes that encode position must be altered. This experimental approach is potentially powerful in investigating whether the expression of homeobox genes is involved in limb patterning.

Investigations of RNA samples from chick limb buds using the polymerase chain reaction show that at least 16 homeobox-containing genes are expressed in the buds (Mackem and Mahon, manuscript in preparation). Two chick homeobox genes have been isolated and sequenced (Wedden *et al.* 1989). One of these, *Ghox 2.1* is expressed in anterior mesenchyme cells of early wing buds. The patterns of expression of several homeobox genes have been mapped in detail in the limbs of mouse embryos and show striking temporal and spatial distributions. Some of these homeobox genes are expressed in both the mesenchyme and the epithelium of the limb bud; others in just the mesenchyme, and in one exceptional case, the expression of a gene homologous to the *Drosophila engrailed* gene, is confined to the ectoderm (Joyner and Martin, 1987). There appear to be three main patterns of mesenchymal expression: expression concentrated in anterior parts of the bud (Oliver *et al.* 1988a,b); that concentrated more posteriorly (Oliver *et al.* 1988a,b; Dollé *et al.* 1989b); and that which becomes confined to the tip of the bud (Hill *et al.* 1989; Robert *et al.* 1989). The group of genes in the *Hox-5* complex which are expressed posteriorly in limb bud mesenchyme also appear to be switched on in a temporal sequence as the limb bud develops (Dollé *et al.* 1989b). In addition,

other homeobox genes are expressed homogeneously throughout limb bud mesenchyme (Duboule and Dollé, 1989; Galliot *et al.* 1989).

XlHbox 1 is a homeobox gene that was first isolated in *Xenopus* and is expressed in the mesenchyme at the anterior margin of the early fore-limb bud (Oliver *et al.* 1988a,b). Antibodies have been prepared to the highly conserved homeoprotein, and reveal the same anterior expression pattern in the mesenchyme of the fore-limb buds of mouse (Oliver *et al.* 1988a,b) and chick embryos (Oliver *et al.* 1990) and in Zebrafish pectoral fins (Molven *et al.* 1990). The role of *XlHbox 1* has been investigated experimentally in chick embryos (Oliver *et al.* 1990). If *XlHbox 1* encodes anterior positional values, then either grafts of polarizing region tissue or applications of retinoic acid that respecify anterior cells to form posterior structures should switch off the gene. However, the results obtained are exactly the opposite of this prediction. The expression domain of *XlHbox 1* expands rather than contracts after manipulations that give rise to additional digits.

The changes in expression of *XlHbox 1* in the mesenchyme correlate with shoulder girdle abnormalities. In the normal wing bud, *XlHbox 1* expression maps to the region that will form the shoulder girdle. Whenever the mesenchymal domain of *XlHbox 1* is expanded, the pattern of the girdle elements immediately abutting the glenoid is affected. In some cases, the coracoid is duplicated (Fig. 4D). Occasionally the skeleton on either side of the glenoid appears symmetrical and the head of the coracoid resembles the head of the scapula (Fig. 4E). Unfortunately, because the way in which the shoulder girdle is specified is unknown, the significance of these changes in pattern is unclear.

Both polarizing region grafts and local application of retinoic acid can switch on expression of *XlHbox 1* in limb mesenchyme cells. The basis for this similar effect on gene expression could be the production of retinoic acid by the polarizing region. The responsiveness of *XlHbox 1* to retinoic acid presents a puzzle. Since retinoic acid is present in higher concentrations posteriorly in the normal wing bud (Thaller and Eichele, 1987), how is expression of the gene confined to the anterior margin? A partial answer to this question is that only anterior limb mesenchyme cells appear to be able to express *XlHbox 1*. When retinoic acid is applied at the apex, the entire anterior part of the bud expresses *XlHbox 1* whereas the posterior half of the wing bud shows no expression (Fig. 4B). In addition, when beads used to apply the retinoid end up posteriorly, the cells immediately surrounding the bead do not express the gene. Furthermore, expression of *XlHbox 1* is cell autonomous rather than position dependent. When the polarizing region, from the posterior part of the wing bud, is grafted anteriorly, the gene is not switched on in the graft. Similarly, when anterior tissue is placed posteriorly, expression is maintained in the graft for up to 24 h (Fig. 4C). Clearly, the role of *XlHbox 1* and the control of its expression are quite complex. It will be interesting to find out how homeobox genes that are expressed either posteriorly

or at the tip of the bud behave when the chick wing bud is manipulated.

Finally, an intriguing position-specific marker has been found in chick wing buds. A monoclonal antibody stains a fan-shaped region of mesenchyme cells in a region of the bud that will give rise to tissue that lies between digits 2 and 3 (Ohsugi and Ide, 1986). When a polarizing region is grafted to the anterior margin to duplicate the pattern of digits, a second patch of antibody staining cells is induced. It is not known whether the same result is obtained when the wing buds are treated with retinoic acid.

Coordination of patterning and morphogenesis

The patterning of the limb is linked to morphogenesis. When a polarizing region is grafted to the anterior margin, the bud widens and tissue is generated to accommodate the additional digits that will develop (Tickle et al. 1975). Retinoic acid application that produces duplicate digits also leads to bud widening. The apical ectodermal ridge lengthens and extends over the anterior part of the bud where the additional digits will form (Lee and Tickle, 1985). The behaviour of the ridge is dose-dependent and when higher concentrations of retinoic acid are applied, the ridge shortens. By varying the dose and position of retinoic acid application, wings with different numbers of digits can be produced. Examination of these buds shows that there is a direct relationship between the length of the apical ridge and the number of digits that will form (reviewed by Brickell and Tickle, 1989). These observations suggest a central role for the apical ridge in controlling the shape of the limb bud.

The changes in ridge length that follow retinoic acid treatment appear to be mediated by the mesenchyme (Tickle et al. 1989). This is shown by recombination experiments (Tickle et al. 1989; Oliver et al. 1990) in which retinoid-treated buds are split into mesenchymal cores and ectodermal hulls, and then recombined with the appropriate tissue from untreated buds. For example, when epithelium taken from buds in which the apical ridge has flattened is recombined with untreated mesenchyme, an apical ridge is re-established in the epithelium by 24 h; and the recombined tissues give rise to a normal set of digits. In contrast, when mesenchyme from treated limb buds is recombined with normal epithelium, the development of the recombination is dictated by the retinoid effect on the mesenchymal component. When mesenchyme is taken from buds that will give truncated limbs, the recombinations do not develop digits; in contrast, when mesenchyme is taken from buds that will give rise to additional digits, the recombinations develop additional digits (Fig. 5A,B). These experiments show that retinoic acid has irreversible effects on the mesenchyme of the bud and suggest that a continuous interaction between mesenchyme and epithelium determines ridge morphology.

The apical ridge is known to be required for bud

outgrowth and stimulates local proliferation of mesenchymal cells in high density cultures (Reiter and Solursh, 1982). Therefore the length of the apical ridge could spatially control cell proliferation in the wing bud and thus define the shape of the bud. The spatial and temporal effects on cell proliferation have been investigated in buds treated with retinoic acid to manipulate ridge length. Three main results support the idea that the ridge plays a central role in controlling cell proliferation (Wilde et al., manuscript submitted). Firstly, in buds treated with retinoic acid to obliterate the ridge, a consistent decrease in cell proliferation occurs after the ridge has flattened; secondly, the effects of retinoic acid on cell proliferation in the limb bud do not closely resemble the effects in culture, suggesting that a direct effect of retinoic acid can be ruled out; and thirdly, the effect of retinoic acid on cell proliferation would appear to be long term, since many of the changes in proliferation take place at a time when the source of retinoic acid can be removed without affecting alterations in morphogenesis and pattern. Another interesting finding to emerge is that the extra tissue generated to accommodate the additional digits is not due simply to a stimulation of cell proliferation. An important contributory factor is that a high rate of proliferation is maintained in regions of the bud where proliferation would normally fall.

The dissection of the effects of retinoic acid on the morphogenetic changes that accompany patterning lend support to a scheme in which a number of steps are involved. The hypothesis is that retinoic acid either directly or indirectly specifies the position of mesenchyme cells in the bud, such specification of mesenchyme cells then determining the interaction of the cells with the apical ridge that maintains its morphology, with the apical ridge then producing a signal that controls local cell proliferation in the bud mesenchyme.

The signals that control epithelial–mesenchymal interactions during vertebrate limb development are unknown. Recently, new clues have emerged that may allow insights into the molecular basis of these interactions. Insertional mutagenesis in mice has led to the sequencing of a gene that appears to be required for bud morphogenesis (Woychik et al. 1985) and growth factors are good candidates for substances that mediate epithelial–mesenchymal interactions.

The isolation of the limb deformity gene is an important advance. In limb buds of the homozygous mutants (ld/ld), the apical ridge is patchy and reduced in length. This results in narrow buds and deficiencies in the pattern across the a–p axis. The gene is apparently expressed in both epithelium and mesenchyme in normal development (Zeller et al. 1989).

Another promising entry into the basis of epithelial–mesenchymal signalling could be provided by a study of growth factors. The growth factors known to be expressed in vertebrate limb development include members of the TGF-β family, IGFs and FGF (Lyons et al. 1989, 1990; Engstrom et al. 1987; Ralphs et al. 1990; Seed et al. 1988). For example, it has been shown recently that members of the TGF-β family are
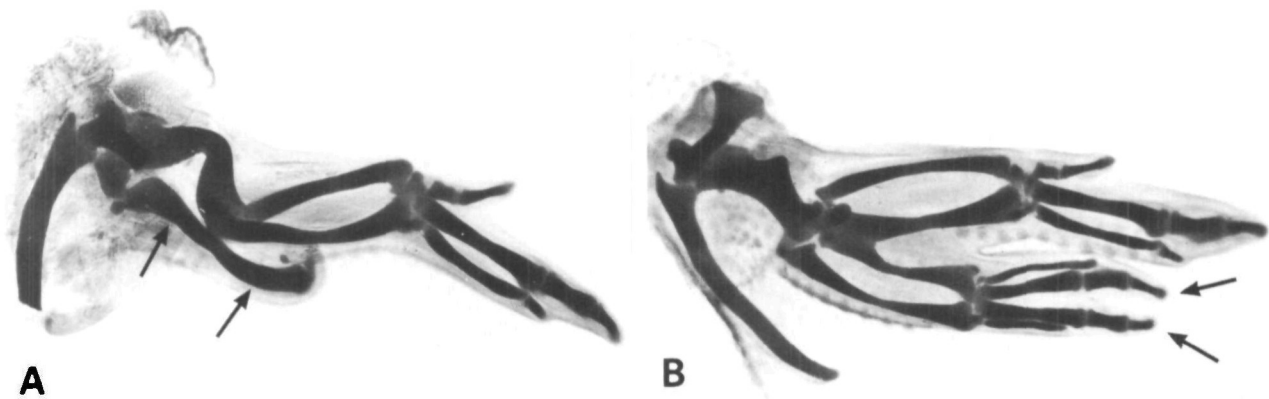
Fig. 5. Recombinations of retinoic acid-treated mesenchyme and epithelial jackets of normal limb buds. The apical ectodermal ridge forms a thickened seam along the distal edge of the jacket. The recombined tissues were grafted to the dorsal surface of a host wing bud to continue development. (A) and (B) show whole mounts of the host wing and skeleton that developed from the recombination. (A) Mesenchyme taken from bud 21 h after implantation of bead soaked in 1 mg ml^{-1} retinoic acid at the apex. In combination with normal epithelium, the mesenchyme has given rise to a cartilage element that resembles the humerus (arrows). No digits have formed. (B) Mesenchyme taken from bud 24 h after implantation of a bead soaked in 0.1 mg ml^{-1} retinoic acid at the anterior margin. In combination with normal epithelium, the mesenchyme has given rise to a mirror-image duplicated pattern of digits, **4334** (arrowed). The normal sequence of digits above developed from the host wing bud.

expressed sequentially in the developing mouse limb buds as chondrogenesis occurs (Lyons *et al.* 1989). However, at early stages, BMP-2A (a bone morphogenetic protein) is expressed in the ventral ectoderm and then becomes localized to the apical ectodermal ridge (Lyons *et al.* 1990).

The distribution of IGFs is also suggestive of a role in epithelial–mesenchymal interactions in the chick wing bud. Immunohistochemical studies show that IGFs are present in undifferentiated chick wing mesenchyme (Ralphs *et al.* 1990). As development proceeds, IGFs are still present at the tip of the limb bud in the progress zone. In prechondrogenic areas, there is no staining with the antibody. It will be interesting to find out whether the distribution of IGFs is affected by retinoic acid treatments that modify morphogenesis of the bud. It may also be relevant that high concentrations of insulin maintain the apical ectodermal ridge in culture (Boutin and Fallon, 1984).

Conclusions

The developing chick limb has provided a framework for analyzing mechanisms of pattern formation in vertebrates. A focus on the limb may allow elucidation of these mechanisms at the cellular and now the molecular level. It seems likely that such a focus may allow a full account of the patterning process in one part of the vertebrate embryo.

The principles uncovered in the developing limb should be generally applicable. Retinoids may be important signalling substances in other parts of the vertebrate embryo in which patterns of connective tissues are generated. For example, application of retinoic acid to chick embryos produces a specific facial defect and patterning and morphogenesis of the facial

primordium that gives rise to the upper beak are inhibited (Tamarin *et al.* 1984). In addition, local application of retinoic acid to the developing spine can induce the formation of additional vertebral structures (S. M. Weale, S. E. Wedden, S. M. Wilde and C. Tickle, unpublished observations). More recently, retinoic acid has been implicated in patterning of the nervous system. It has been suggested that retinoic acid could be involved in the a–p specification of the neural tube (Durston *et al.* 1989). Furthermore the floor plate of the neural tube can generate retinoic acid from retinol (Wagner *et al.* 1990). This may be the basis of the signalling of the floor plate that regulates some aspects of neuronal differentiation and axonal guidance (Jessell *et al.* 1989). Recent investigations reveal striking temporal and spatial patterns of expression of retinoic acid receptors and cellular binding proteins in the face, spine and nervous system of vertebrate embryos (Ruberte *et al.* 1990; Perez-Castro *et al.* 1989; Osumi-Yamashita *et al.* 1990; Maden *et al.* 1990). These findings reinforce the idea that retinoic acid plays a role in the development of these parts of the embryo, in addition to the limb. Finally, it is also interesting that some of the homeobox genes expressed in limb buds and potentially regulated by retinoic acid are also expressed in these regions of the body. For example, some of the homeobox genes expressed in the limb are also expressed along the main body axis (Dollé and Duboule, 1989) and others are expressed in both the limb bud and facial primordia (Hill *et al.* 1989; Robert *et al.* 1989). Taken together then, glimmerings of a picture of how patterning may operate at the molecular level in vertebrates are beginning to emerge.

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