

## The role of the polarizing zone in the pattern of experimental chondrogenesis in the chick embryo interdigital space

DOMINGO MACÍAS\* and YOLANDA GAÑÁN

*Department of Morphological Sciences and Cellular and Animal Biology, University of Extremadura, Badajoz, Spain*

**ABSTRACT** We have previously shown that removal of the apical ectodermal ridge of the third interdigital space of the chick leg bud at stages 28 and 29 is followed by the appearance of ectopic cartilage, which in the course of development gives rise to extra digits. These *in vivo* studies suggest that the pattern of skeletal morphogenesis in the limb depends on the inhibitory effect of the ectoderm. In the present study we tested whether zone polarizing activity (ZPA) exerted an effect on the pattern of experimental chondrogenesis in the interdigital space of the leg bud in stage 29 HH chick embryos. A small fragment of tissue from the ZPA in chick embryos in which ZPA activity was most intense was grafted onto the interdigital space in which chondrogenesis had previously been experimentally induced. No significant changes were observed in the course of differentiation of the recipient interdigital spaces with ZPA grafts, leading us to conclude that the graft failed to modify the morphogenetic fate of interdigital tissue.

**KEY WORDS:** *limb morphogenesis, chondrogenesis, zone of polarizing activity (ZPA), chick embryo*

The mesoderm of the limb bud contains at least two clearly differentiated populations of cells. The population derived from the somatopleural mesoderm gives rise to skeletal elements and connective tissue, while that derived from the somitic mesoderm forms the condensations of the premuscular masses in the limb (Chevalier *et al.*, 1977; Christ *et al.*, 1977).

The pattern of morphogenesis in the limb seems to be mediated by a series of information-bearing mechanisms that control limb growth in all three directions in space. In recent years, some authors (Sulursh *et al.*, 1981; Sulursh 1984) have shown that the pattern of morphogenesis in the limb skeleton may depend on an intrinsic tendency of the mesenchyme to form cartilage, together with an inhibitory effect of the ectoderm. In transverse sections of developing limbs, these authors observed that the nuclei of chondrogenesis consistently kept a critical distance from the ectoderm covering the anlage, the space between them being occupied by undifferentiated mesenchymal cells. Recent *in vivo* studies by Hurlé and Gañán (1986, 1987) provide strong support for this hypothesis. These authors reported that removal of the apical ectodermal ridge (AER) from the third interdigital space in chick embryo limb bud led not only to ectopic chondrogenesis, but also to the formation of extra digits, thus altering the antero-posterior pattern of the limb by transforming an interdigital space into a digit.

Classically, the antero-posterior pattern of the developing limb was thought to be controlled by a diffusible factor originating from a small region on the posterior edge of the limb anlage, and termed

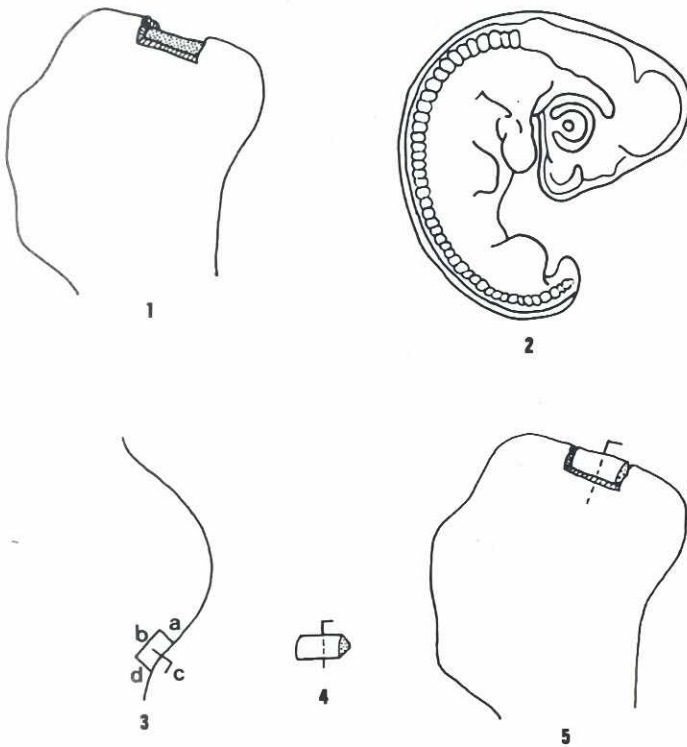
the zone of polarizing activity (ZPA) (Tickle *et al.*, 1975; Tickle 1980). However, since the period studied by Hurlé and Gañán (1987) did not include the time when the hypothetical factors (including the ZPA) of morphogenetic control are thought to act (Hinchliffe and Gumpel-Pinot, 1981; Hinchliffe and Griffiths, 1984; Hinchliffe *et al.*, 1984), alternatives to the classical morphogenetic factors must be considered. An especially attractive notion is that the ectoderm may somehow regulate the pattern of chondrogenesis, as suggested by Sulursh *et al.* (1981) and Sulursh (1984).

The present study was designed to determine whether the ZPA influences the pattern of experimental chondrogenesis in the interdigital space. We used small fragments of tissue from the ZPA or the anterior edge of the limb bud of chick embryos in the stage of maximum ZPA activity, and grafted them onto the interdigital space of chick embryos in which we had previously induced experimental chondrogenesis. Our results show that the ZPA graft in the interdigital space failed to modify the pattern of experimental chondrogenesis.

We have previously observed that such grafts produce incomplete epithelization in a number of experimental cases. This led us to investigate whether the pattern of chondrogenesis in grafts of limb fragments varied in response to the degree of epithelization of the

*Abbreviations used in this paper:* ZPA, zone of polarizing activity; HH, stages of Hamburger and Hamilton; AER, apical ectodermal ridge; SEM, scanning electron microscope

\*Address for reprints: Departamento de Ciencias Morfológicas, Facultad de Medicina, Universidad de Extremadura, E-06071 Badajoz, SPAIN. FAX: 34-24-23.63.04



**Fig. 1. Schematic diagram of the surgical procedure used for limb grafts.** 1. Ablation of the AER from the third interdigital space. 2. HH stage 19-20 donor embryo. 3. Fragment of ZPA removed from donor embryo limb. 3a. Post-axial incision perpendicular to the proximo-distal axis. 3b. Longitudinal incision parallel to the posterior edge of the limb. 3c. Angled platinum needle. 3d. Incision perpendicular to the proximo-distal axis and parallel to the first incision. 4. Isolated fragment of ZPA. 5. Experimental host limb with the ZPA graft in the site from which the AER was removed.

grafts. Our findings confirmed that zones devoid of ectoderm produced chondrogenesis with rounded or ovoid appearance, whereas the morphology of the epithelized distal portion formed growing distal phalanges.

We analyzed a total of 40 chick embryos that survived surgery for more than 72 h. In 25 of these embryos the graft came from the posterior edge of the limb bud which contained ZPA tissue, and in 15 remaining embryos the graft consisted of tissue from the anterior half of the limb bud. In half of the 40 specimens the graft was from the wing bud, and in the other half the graft was the leg bud.

No significant differences were found between the effects of grafts from the ZPA and the anterior edge of the anlage, or between wing and leg grafts. The phenotype of the interdigital tissue and the morphogenesis of the neighboring digits did not appear to be modified by the ZPA.

Three different kinds of post-graft development were distinguishable. In 10 embryos the graft showed little subsequent development, and was identifiable only as a small mass of tissue associated to the platinum microneedle used to hold the graft in place. As Figs. 2 and 3 illustrate, the graft was almost completely separate from the host although the platinum needle was permanently covered with lining cells. Whole block staining for cartilage

revealed some indication of chondrogenesis, and given the unchanging appearance of the graft and its minimal union with the host tissue, we classified these embryos as cases of failure of the surgical technique.

In 15 embryos the graft continued to develop, forming nodules of chondrogenesis. However, the interdigital membrane underwent normal regression, although in some cases the process of reabsorption was slightly delayed. Morphologically, all grafts in this group contained cartilaginous elements similar to the distal components of the autopodium (Fig. 4). An interesting feature was that the ectodermal covering was incomplete in the most proximal portion of the surface of the graft (Fig. 5); in this zone the chondrogenic nucleus was consistently more prominent (Fig. 6). In all 15 specimens the mesenchyme of the graft showed a somewhat reduced degree of continuity with the host limb mesenchyme, which nevertheless was probably sufficient to sustain the passage of blood vessels.

The graft in the remaining 15 embryos showed clear signs of growth, accompanied by a thin interdigital membrane similar to that seen in membranous syndactylia (Fig. 7). As Fig. 8 shows, the graft was limited to the distal part of the membrane, and from the location of the platinum needle we assumed that the growing structure originated from the graft.

The incidence of ectopic digits was lower than seen after removing the apical ectodermal ridge without subsequent grafting. The extra digits that formed were morphologically similar to those observed by Hurle and Gañan (1987) and Hurle *et al.* (1989) after they removed the apical ectodermal ridge or wedges of tissue from the third interdigital space of the limb bud, respectively (Fig. 9).

In none of our embryos did we observe significant alterations in the digits next to the interdigital space, regardless of the fate of the graft.

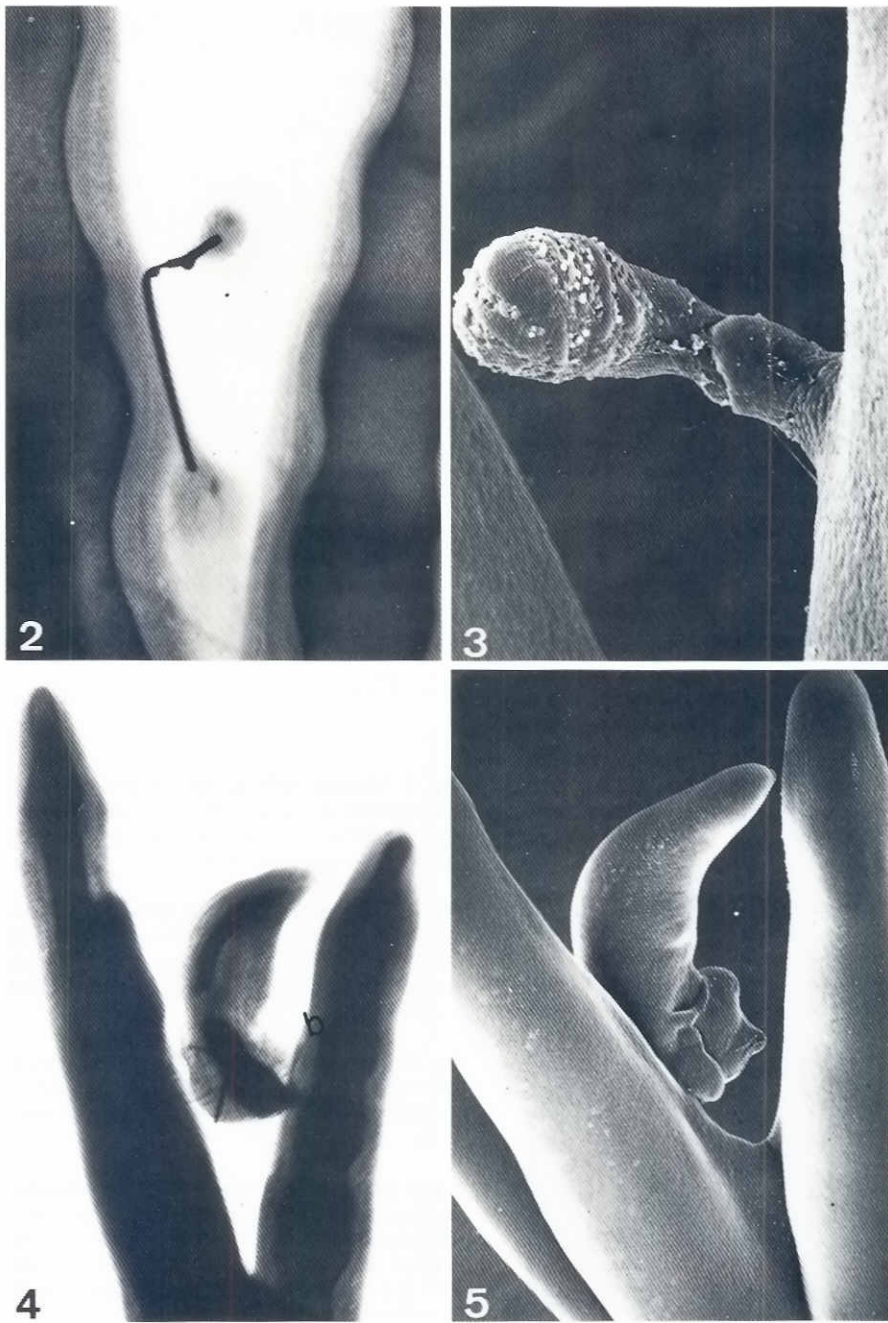
In the chick limb bud, the zone of polarizing activity (ZPA) plays an important role in pattern formation. Numerous studies in the literature have cited the ZPA as the source of a morphogen (Tickle *et al.*, 1975; MacCabe and Parker, 1976) possibly related with vitamin A (Tickle *et al.*, 1982; Summerbell, 1983; Maden, 1985; Tickle *et al.*, 1985; Eichele and Thaller, 1987; Wilde *et al.*, 1987; Hinchliffe, 1989) that governs the antero-posterior pattern of the bud, and in particular the pattern of the digits.

Our findings seem to show that the interdigital tissue did not respond to the hypothetical morphogen produced by the ZPA, nor were there any alterations in the neighboring digits. From previous ectoderm removal experiments (Hurle and Gañan, 1986 and 1987; Hurle *et al.*, 1989), we know that the pattern of cartilage differentiation at the tip of a stage-29 leg bud is not yet fully determined.

These observations suggest that the morphogen from the ZPA does not act by directly controlling cartilage differentiation.

The fact that the effect of the ZPA on limb morphogenesis is manifested only by ZPA grafts made in the anterior edge of the limb bud, whereas their removal did not affect normal morphogenesis (MacCabe *et al.*, 1973; Saunders, 1977), suggests that the effect of the ZPA may be due to an interaction with the anterior half of the limb, possibly concurrent with development of the AER.

Some of our observations of the fate of ZPA grafts seem to be informative about the mechanisms responsible for regression of the interdigital tissue. Hurle and Fernández-Terán (1983) suggested that the eventual disappearance of the interdigital membrane under normal conditions results from the interaction between the regressing mesenchyme and the ectoderm covering the interdigital spaces.



**Fig. 2.** Alcian blue staining shows a small chondrogenic nodule at the end of the platinum needle in the interdigit of a leg bud three days after of ZPA was grafted at stage 29. X 125.

**Fig. 3.** Detail of the experimental limb in Fig. 2 under scanning electron microscope showing the chondrogenic nodule and the epithelized platinum needle. X 500.

**Fig. 4.** Experimental leg bud at stage 29 four days after a fragment of ZPA from the leg bud of an HH 19-20 embryo was grafted. Alcian blue staining shows skeletal growth in the graft, and absence of the interdigital membrane. X 75.

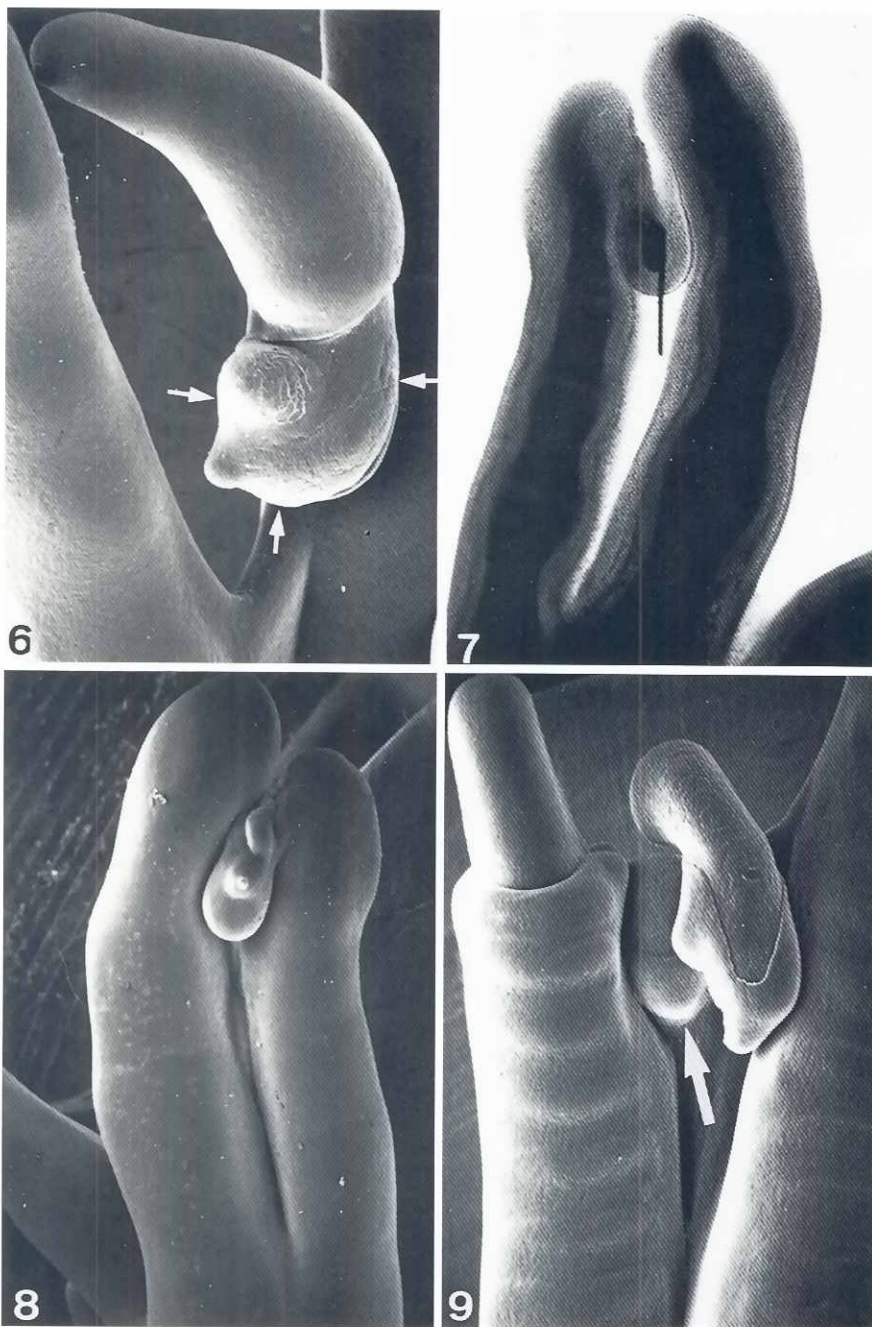
**Fig. 5.** Dorsal view of the same experimental limb as in Fig. 4 under scanning electron microscope shows growth of the graft adhered to the third digit, together with the absence of proximal epithelization. X 75.

According to these authors, cell death may modify the interdigital extracellular material, a change which may in turn bring about detachment of the ectoderm.

Our findings show that when a fragment of tissue not destined to die was grafted onto the distal edge of the interdigital space, detachment of the interdigital tissue from the host was prevented, giving rise to membranous syndactylia even though most of the mesenchyme had disappeared due to cell death. This lends support to the notion that disappearance of the interdigital tissue requires a free edge from which the cells can become detached.

Another significant observation of this study was that the ZPA graft continued to grow. This agrees with the findings of Brand *et al.* (1985), who reported that the graft produced a programmed zone in the donor region. Thus grafts from the prospective wing zone developed into wing bud, whereas grafts from the leg gave rise to leg bud. In both cases the skeletal elements formed by the graft were distal limb components, whose number depended on the size of the original graft.

The fact that many ZPA grafts in our study did not degenerate, but went on to form cartilaginous elements, suggests that no necrotic



**Fig. 6. Ventral view under scanning electron microscope** of the growth of the graft shown in Figs. 4 and 5, showing the absence of proximal epithelization (arrows). X 100.

**Fig. 7. Experimental limb at stage 29** four days after a fragment of ZPA from the wing bud at stage 19-20 was grafted. Alcian blue staining reveals growth of the free edge of the persisting interdigital membrane. X 75.

**Fig. 8. Dorsal view under SEM** of the same experimental leg bud as in Fig. 7 showing the interdigital membrane of the third interdigital space. X 75.

**Fig. 9. Scanning electron micrograph of a chick embryo at stage 29** five days after a fragment of ZPA from chick embryo leg bud at stage 19-20 was grafted. Note the ectopic digit (arrow) associated with growth of the graft and persistence of the interdigital membrane. X 75.

factor diffused out from the degenerating interdigital mesenchyme and grafted tissue.

A final feature observed in our study was that the shape of the tissue grafted into the interdigit appeared related with its degree of epithelization. As mentioned above, in the course of development the grafts undergo progressive growth and chondrogenesis. Analysis with SEM revealed the occurrence of areas in the proximal part of the graft with defective epithelization. A constant feature of these areas was the presence of a cartilaginous nucleus without surrounding mesenchyme and lacking a definite shape. These findings

again support Solursh's suggestion (1984) that the limb ectoderm controls the spatial pattern of chondrogenesis by producing an antichondrogenic factor that spreads inward from the periphery to the nucleus of the limb.

### Experimental Procedures

We used White Leghorn chick embryos that were incubated at 38.5°C. Host embryos were used at Hamburger and Hamilton's (HH) (1951) stage 29, and donor embryos were used at HH stages 19-20.

Surgical procedures were performed under a binocular dissecting microscope. At stage 29 we first ruptured the amniotic membrane with microsurgery tweezers to expose the right limb, the AER was removed from the third interdigital space with a fine tungsten needle, taking care not to damage the neighboring digits (Fig. 1). Under a second dissecting microscope, we prepared the donor embryo, which was placed in a petri dish with Ringer's avian solution. The limb was exposed and a small fragment containing the ZPA was taken from the posterior edge using a fine tungsten needle. A post-axial incision was first made perpendicular to the proximo-distal axis of the limb, then a longitudinal incision was made parallel to the posterior edge of the limb. An angled piece of platinum microneedle (Goodfellow Metals, Cambridge) measuring 0.025 mm in diameter was guided with microsurgery tweezers into the fragment, and the ZPA fragment was freed with a third incision made perpendicular to the proximo-distal axis and parallel to the first incision. The tissue fragment was picked up with the platinum microneedle, which was in turn held with tweezers, and transferred to the site in the host leg bud from which the apical ectodermal ridge had been removed (Fig. 1).

In a second group of embryos the graft was taken from the preaxial region of the donor according to the procedure just described for the ZPA fragment. After surgery, the eggs were returned to the incubator for 72 h, after which time the embryos were sacrificed for light and electron microscopic observation. For light microscopic analysis the limbs were fixed in Bouin liquid and stained with alcian blue 8 GX (Gurr) according to the technique described by Ojeda *et al.* (1970), then rinsed in xylol. After the skeletal elements were analyzed, the limbs were processed for scanning electron microscopic observation.

#### Acknowledgments

This work was supported by a grant from the DGICYT (PB 89-0493). Thanks to Ms. Karen Shashok for translating the original manuscript into English.

#### References

- BRAND, B., CHRIST, B. and JACOB, H.J. (1985). An experimental analysis of the development capacities of distal parts of avian leg buds. *Am. J. Anat.* 173: 321-340.
- CHEVALLIER, A., KIENY, M. and MAUGER, A. (1977). Limb-somite relationship: origin of the limb musculature. *J. Embryol. Exp. Morphol.* 41: 245-258.
- CHRIST, B., JACOB, H.J. and JACOB, M. (1977). Experimental analysis of the origin of the wing musculature in avian embryos. *Anat. Embryol.* 150: 171-186.
- EICHELE, G. and THALLER, C. (1987). Characterization of concentration gradients of a morphogenetically active retinoid in the chick limb bud. *J. Cell Biol.* 105: 1917-1923.
- HAMBURGER, V. and HAMILTON, H.L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* 88: 49-92.
- HINCHLIFFE, J. R. (1989). Evolutionary aspects of the developmental mechanism underlying the patterning of the pentadactyl limb skeleton in birds and other tetrapods. In *Trends in Vertebrate Morphology* (Eds. H. Splechna and H. Hilgers). Gustav Fisher Verlag, pp. 226-229.
- HINCHLIFFE, J.R. and GRIFFITHS, P.J. (1984). Experimental analysis of the control of cell death in chick limb bud development. In *Cell Ageing and Cell Death* (Eds. I. Davies and D.C. Sigee). Cambridge University Press, Cambridge, pp.223-242.
- HINCHLIFFE, J.R. and GUMPEL-PINOT, M. (1981). Control of maintenance and antero-posterior differentiation of the anterior mesenchyme of the chick wing bud by its posterior margin (the ZPA). *J. Embryol. Exp. Morphol.* 62: 63-82.
- HINCHLIFFE, J.R., GUMPEL-PINOT, M., WILSON, D.J. and YALLUP, B.L. (1984). The prospective skeletal areas of the chick wing bud: their location and time of determination in the limb field. In *Matrices and Cell Differentiation* (Eds. R.B. Kemp and J.R. Hinchliffe). Alan R. Liss Inc., New York, pp.453-470.
- HURLE, J.M. and FERNANDEZ-TERAN, M.A. (1983). Fine structure of the regressing interdigital membranes during the formation of the digits of the chick embryo leg bud. *J. Embryol. Exp. Morphol.* 78: 195-209.
- HURLE, J.M. and GAÑAN, Y. (1986). Interdigital tissue chondrogenesis induced by surgical removal of the ectoderm in the embryonic chick leg bud. *J. Embryol. Exp. Morphol.* 94: 231-244.
- HURLE, J.M. and GAÑAN, Y. (1987). Formation of extra-digits induced by surgical removal of the apical ectoderm ridge of the chick embryo leg bud in the stages previous to the onset of interdigital cell death. *Anat. Embryol.* 176: 393-399.
- HURLE, J.M., GAÑAN, Y. and MACIAS, D. (1989). Experimental analysis of the *in vivo* chondrogenic potential of the interdigital mesenchyme of the chick leg bud subjected to local ectodermal removal. *Dev. Biol.* 132: 368-374.
- MacCABE, J.A. and PARKER, B.W. (1976). Polarizing activity in the developing limb of the Syrian hamster. *J. Exp. Zool.* 195: 311-317.
- MacCABE, J.A., SAUNDERS, J.W. and PICKETT, M. (1973). The control of the antero-posterior and dorso-ventral axis in embryonic chick limbs constructed of dissociated and reaggregated limb bud mesoderm. *Dev. Biol.* 31: 323-335.
- MADEN, M. (1985). Retinoids and the control of pattern in limb development and regeneration. *Trends Genet.* 1: 103-107.
- OJEDA, J.L., BARBOSA, E. and GOMEZ-BOSQUE, P.G. (1970). A rapid method for selective staining of whole chicken embryos. *Stain Technol.* 42: 262-264.
- SAUNDERS, J.W. (1977). The experimental analysis of chick limb bud development. In *Vertebrate Limb and Somite Morphogenesis* (Eds. D.A. Ede, J.R. Hinchliffe and M. Balls). Cambridge University Press, Cambridge, pp. 1-24.
- SOLURSH, M. (1984). Ectoderm as a determinant of early tissue patterns in the limb bud. *Cell Differ.* 15: 17-24.
- SOLURSH, M., SINGLEY, C.T. and REITER, R.S. (1981). The influence of epithelia on cartilage and loose connective tissue formation by limb mesenchyme cultures. *Dev. Biol.* 86: 471-482.
- SUMMERBELL, D. (1983). The effect of local application of retinoid acid to the anterior margin of the developing chick limb. *J. Embryol. Exp. Morphol.* 78: 269-289.
- TICKLE, C. (1980). The polarizing region and limb development. In *Development in Mammals*, Vol. 4 (Ed. M.H. Johnson). Elsevier/North Holland Biomed. Press, Amsterdam, pp.101-136.
- TICKLE, C., ALBERTS, B., WOLPERT, L. and LEE, J. (1982). Local application of retinoic acid to the limb mimics the action of the polarizing region. *Nature.* 296: 564-566.
- TICKLE, C., LEE, J. and EICHELE, G. (1985). A quantitative analysis of the effect of retinoic acid on pattern formation in the developing chick wing. *Dev. Biol.* 109: 82-95.
- TICKLE, C., SUMMERBELL, D. and WOLPERT, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* 254: 199-202.
- WILDE, S.M., WEDDEN, S.E. and TICKLE, C. (1987). Retinoids reprogramme pre-bud mesenchyme to give changes in limb pattern. *Development* 100: 723-733.

Accepted for publication: January 1991