

I am submitting herewith a dissertation by Jane S. Geduspan entitled "Control of Dorso-Ventral Patterns of Differentiation in the Developing Chick Wing." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.


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CONTROL OF DORSO-VENTRAL PATTERNS OF DIFFERENTIATION IN THE
DEVELOPING CHICK WING

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ABSTRACT

The influence of limb ectoderm on dorso-ventral pattern in the chick wing was studied by recombining stage 14-21 limb mesoderm with the same stage ectoderm in dorso-ventrally reversed orientation. Fully-developed wings obtained from stage 15-21 donor embryos have at their distal half dorso-ventral polarity conforming to the reversed ectoderm. The ectodermal effect is generally observed as a bidorsal feather pattern at the autopod and an almost complete dorso-ventral reversal of muscle and skeletal patterns. At the prelimb stages (younger than stage 15), however, control of dorso-ventral patterns in the wing resides in the mesoderm. Recombination experiments at stage 14, with dorso-ventrally reoriented ectoderm, result in wings with mesodermal dorso-ventral polarity. At the onset of limb development at stage 15, some of the control shifts to the ectoderm. By stage 16, the ectoderm has acquired dorso-ventral information and can impose this polarity on the patterns of mesodermal differentiation in the distal regions of the wing.

The dorso-ventral information in the ectoderm comes from the mesoderm which transfers this information to the overlying ectoderm between stage 14-16. The initial dorso-ventral information in the ectoderm is not stable and can be "reprogrammed" by stage 14 mesoderm. Subsequently, there is a gradual stabilization of the ectodermal information. At the same time the mesoderm loses its capacity to "reprogram" the dorso-ventral information in the ectoderm.

Dorso-ventral differentials in presumptive limb mesoderm were eliminated by superimposition of two limb fields with opposing dorso-ventral axes. This interfered with formation of the ectodermal ridge and subsequent limb outgrowth. The dorso-ventral differences, however, are not necessary for ridge induction since dorsal and ventral limb mesoderms can promote formation of the ridge and fully-developed wings after transplantation to the dorsal limb or flank regions. The dorso-ventral differentials in the limb field are expressed as limb-promoting activity in the dorsal region and an inhibitory influence on limb initiation coming from the ventral limb region. The boundary between these two opposing influences may be significant in determining the site of ectodermal ridge formation.

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GENERAL INTRODUCTION

The initial indications of limb development in the chick embryo are paired condensations of the somatopleural mesoderm ventral to the somites (somites 15-20 for the wing and somites 26-32 for the leg). The limb-forming mesoderm has a dual origin, somatic mesoderm from the body wall and somitic mesoderm migrating into the presumptive limb from the adjacent somites. The mesodermal condensations increase in thickness and appear as recognizable swellings from the lateral body wall. Each thickening is covered by ectoderm, the distal border of which is thickened to form the apical ectodermal ridge (AER). As the limb bud grows, differentiation occurs progressing in a proximal to distal direction. The somatopleural mesoderm gives rise to the skeleton, connective tissues, tendons, and vasculature, while the somitic mesoderm forms the intrinsic muscles in the limb (Chevalier et al., 1977; Christ et al., 1977; Chevalier et al., 1978; Kieny and Chevalier, 1979). The ectoderm forms feathers or scales in the adult wing or leg.

The prospective limb-forming regions in the chick embryo are determined early in development, long before the primordia are recognizable. At the very early stages of development, the axial relations in the limb-forming regions are already established with the antero-posterior axis determined earlier than the dorso-ventral axis (Chaube, 1959). Transplants of presumptive limb tissues to the flank of host embryos at stages as early as eleven somites are capable of self differentiation and form limbs with all the axes conforming to

the graft rather than the host (Hamburger, 1938; Chaube, 1959). At the early stages of development, the self-differentiating capacity of the prospective limb is dependent on influences from the adjacent somites. The limb mesoderm, however, becomes independent of the somitic influence after the thirteen-somite stage (Kieny, 1969; Pinot, 1970).

During differentiation in the developing limb, a spatially defined pattern of structures emerges organized according to the antero-posterior, dorso-ventral, and proximodistal axes. This is the result of the precise control of spatial patterns of differentiation within the mesoderm and ectoderm. The control of these patterns, particularly the dorso-ventral pattern, is one of the least understood areas of developmental biology and is the topic of investigation of the research reported here.

The morphogenetic pattern along the antero-posterior axis in the normal wing is dependent on a small region of the mesoderm at the posterior border of the limb bud, the zone of polarizing activity (ZPA). The tissue when transplanted to an anterior position in the host limb buds promotes the development of supernumerary limbs with their posterior borders always adjoining the grafts. In such grafts, a secondary proximodistal axis is established indicated by increased thickness of the ectodermal ridge adjacent to the ZPA marking the region of the supernumerary outgrowth (Saunders and Gasseling, 1968; MacCabe et al., 1973). The supernumerary structures formed show duplications of skeletal elements, muscles, and tendons (Shellswell and Wolpert, 1977). The polarization of the normal limb along the antero-posterior axis may require the presence of the polarizing

tissue (for contrary views see Fallon and Crosby, 1975; Saunders, 1977). The excision of the posterior half of the wing bud (including the highest point of ZPA activity) causes necrosis of the distal half of the remaining anterior mesenchyme and regression of the ectodermal ridge. The anterior half of the wing develops poorly, usually lacking anterior structures (Hinchliffe and Gumpel-Pinot, 1981). The ZPA can also polarize limb structures that develop from dissociated limb mesoderm enclosed by limb ectoderm (MacCabe et al., 1973).

Early experiments by Saunders (1948) show that limb outgrowth and elaboration of structures along the proximodistal axis is dependent on an ectodermal thickening at the distal border of the limb bud, the apical ectodermal ridge (AER). Surgical removal of the ectodermal ridge (Saunders, 1948; Summerbell, 1974) or its absence in wingless mutants (Zwilling, 1956a; Carrington and Fallon, 1984) result in limbs with distal deficiencies. Likewise, grafting an additional ridge to the limb bud results in the development of a supernumerary outgrowth (Zwilling, 1956b; Saunders et al., 1976). The initial formation of the ectodermal ridge is dependent on inductive influence from the underlying mesoderm. Prior to the formation of the limb bud, presumptive wing bud mesoderm grafted to the flank induces the healing flank ectoderm to form a ridge and develops into a typical wing (Kieny, 1960; Dhouailly and Kieny, 1972; Saunders and Reuss, 1974). The ridge induction period is brief, the limb mesoderm gradually losing the capacity to induce a ridge after the appearance of the limb bud (Kieny, 1968; Saunders and Reuss, 1974). The mesoderm also maintains the ridge in its active, thickened configuration (Zwilling

and Hansborough, 1956; Zwillling, 1974). The influence of the ectodermal ridge on the underlying mesoderm is permissive rather than instructive. Exchanges of limb ectoderm between the wing and the leg buds have no effect on the pattern of development of the wing or the leg (Zwillling, 1955). Young ectoderm replaced on a limb mesoderm has the same outgrowth-promoting activity as an older ectoderm (Rubin and Saunders, 1972). The pattern, therefore, along the proximodistal axis is determined by the mesoderm.

Very little is known about the control of dorso-ventral patterns. The specification of pattern along this axis is suggested to be under the control of the limb mesoderm. Grafts of presumptive limb mesoderm to the flank, covered by regenerated flank ectoderm, develop into limbs with the dorso-ventral polarity conforming to the mesoderm, regardless of their orientation on the host (Kieny, 1968; Dhouailly and Kieny, 1972; Saunders and Reuss, 1974). In situ, limb mesoderms combined with flank ectoderms in dorso-ventrally reversed or normal orientation form wings with normal (mesodermal) dorso-ventral polarity (Carrington and Fallon, 1984).

Results of tissue recombination experiments, however, demonstrate that the limb ectoderm is polarized dorso-ventrally and can impose this polarity on the distal regions of the limb. In tissue recombinations with the leg, the dorso-ventral reorientation of the ectoderm changes the scale pattern of the foot to become bidorsal, dorso-ventrally reversed, or in some cases biventral. The mesodermal structures may be affected based on the curvature of the toes and flexion of the tibiotarsal joint (Pautou and Kieny, 1973; MacCabe et

al., 1974; Pautou, 1977). That the ectoderm can affect the development of mesodermal structures is further implied by the abnormalities that develop in the humerus or the appearance of ectopic cartilages following reorientation of blocks of limb ectoderm and subjacent mesoderm (Stark and Searls, 1974; Searls, 1976). Limb mesoderm without dorso-ventral differentials, as in dissociated and reaggregated limb mesenchyme packed into a limb ectoderm, can develop into limbs with their dorso-ventral polarity conforming with the ectoderm (MacCabe et al., 1973). These results suggest that the limb ectoderm may participate in the determination of dorso-ventral pattern in the limb.

The experimental data on the dorso-ventral polarity in the chick limb do not convincingly demonstrate that the ectoderm plays a significant role in the establishment of this axis. Much of the observations are based on the pattern of epidermal structures, feathers or scales. No in vivo effect has been reported on the mesodermal derivatives, i.e., muscles and skeletal elements. There is a general assumption that pattern along this axis is under mesodermal control (Bryant and Iten, 1975; Bryant et al., 1981) based on results of experiments with amphibian limb development.

The main thrust of this work is to define the relative roles of mesoderm and ectoderm in the dorso-ventral polarization of the chick wing. The approach used here has been:

(a) to determine the extent of ectodermal influence on dorso-ventral polarity, i.e., whether its effects extend to the muscles and skeletal elements as well as epidermal structures.

(b) to examine the possibility that dorso-ventral information exchange occurs between mesoderm and ectoderm at the early stages of limb development.

(c) to study the significance of dorso-ventral differentials in the limb mesoderm in relation to ectodermal ridge induction and limb outgrowth.

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PART I

THE ECTODERMAL CONTROL OF MESODERMAL PATTERNS OF
DIFFERENTIATION IN THE DEVELOPING CHICK WING

Introduction

The control of three dimensional pattern formation in the developing and regenerating vertebrate limb has been under intensive investigation in recent years (see for reviews, Maden, 1981; Stocum and Fallon, 1982; Hinchliffe and Gumpel-Pinot, 1983; Holder, 1984; Javois, 1984; Solursh, 1984a). The main thrust of these investigations has been to examine the relative roles of limb mesoderm and ectoderm on pattern formation and the development of models to account for the emergence of pattern in the limb, primarily the pattern of mesodermal differentiation into muscles and skeletal elements. For the chick embryo in particular, these studies have demonstrated that the control of both antero-posterior and proximo-distal patterns resides in the mesodermal component of the limb. No role has been attributed to the ectoderm other than the importance of the ectodermal ridge for continued proximal to distal outgrowth and elaboration of distal structures in the limb. The spatial pattern of differentiation along the proximo-distal axis, however, is a function of the limb mesoderm (Rubin and Saunders, 1972).

There is conflicting evidence on the relative roles of mesoderm and ectoderm in the development of dorso-ventral patterns in the chick limb. Grafts of presumptive limb mesoderm to the flank of host embryos induce the healing flank ectoderm to form ridge (Kieny, 1960; Dhouailly and Kieny, 1972; Saunders and Reuss, 1974). Regardless of their orientation relative to host's axes, the resulting limbs have their dorso-ventral polarity conform with the donor mesoderm. In situ,

stage 15 presumptive wing bud mesoderm recombined with flank ectoderm in dorso-ventrally reversed or normal orientation give rise to wings with their dorso-ventral differentials also corresponding with the mesoderm (Carrington and Fallon, 1984). Some studies however have indicated that the limb ectoderm can influence the dorso-ventral polarity of the distal regions of the limb. Limb mesoderm recombined with dorso-ventrally reversed ectoderm when grafted on host embryos develop into limbs with the polarity of the epidermal structures reversed. In tissue recombinations with the leg, the scale pattern of the foot is altered to become either bidorsal, dorso-ventrally reversed, or in a few cases biventral (MacCabe et al., 1973; Patou and Kieny, 1973; MacCabe et al., 1974). In the absence of dorso-ventral differentials in the limb mesoderm, as in dissociated and reaggregated limb mesoderm packed into limb ectoderm, the dorso-ventral polarity of the epidermal structures conform with the ectoderm (MacCabe et al., 1973; Patou, 1977). These findings suggest that the limb ectoderm is not a passive component during limb development but appears to have an important role in the establishment of the limb's dorso-ventral differentials.

The present study determines the extent of ectodermal influence on the dorso-ventral polarity of the mesodermal components of the chick wing, in particular muscles and skeletal elements. Results of recombination experiments show that the dorso-ventral reversal of the limb ectoderm relative to the mesoderm causes reversal of muscle and cartilage patterns in the distal region of the wing. This ectodermal

effect is observed when the ectoderm is reoriented after, but not before, stage 15.

Materials and Methods

Fertile White Leghorn eggs were obtained from the Department of Animal Science at the University of Tennessee and incubated in a humidified incubator at 38°C. On the second or third day of incubation, after removal of 2 ml albumin from each egg, windows were cut in the shell and then sealed with Parafilm (American Can Co., Greenwich, Connecticut). The eggs were returned to the incubator and allowed to develop until the desired stages were obtained.

Right and left presumptive wings or wing buds were excised from stage 14-21 embryos and transferred to cold phosphate-buffered saline (PBS). After rinsing in calcium-magnesium free PBS (CMF-PBS), right prospective wings or wing buds, used as mesoderm donors, were incubated at 38°C in 1% EDTA (in CMF-PBS) for 20 minutes. The presumptive limbs were then stripped of their ectoderm and returned to cold PBS until ectoderm donors were ready.

Left prospective limbs, used as ectoderm donors, were transferred to a Horse serum-Tyrode's solution (1:2 v/v) after incubation in 1% trypsin in CMF-PBS for 1 1/2-2 hours at 4°C. The ectoderm was separated from its mesoderm and immediately recombined with the right wing bud mesoderm in dorso-ventrally reversed orientation. Recombinant limbs were left at room temperature until the ectoderm closely adheres to the mesoderm. The composite limb buds or presumptive limbs were

grafted to the flank of stage 19-21 host embryos and allowed to develop for ten days.

On the tenth day of incubation, host embryos were sacrificed and recombinant wings recovered. Well-developed wings were evaluated for evidence of dorso-ventral reversal by examination of the feather follicle pattern and then fixed in Bouin's fixative. At least four wings from each stage were paraffin embedded, serially sectioned at 7 μ m, and stained with Milligan's Trichome stain to examine muscle and cartilage patterns. Muscles were identified according to their origin and insertion (Sullivan, 1962; Shellswell, 1977) and considered reversed if their origin and insertion correspond with a normal muscle but found at a site opposite to their normal position (i.e., dorsal muscle at the ventral region and vice versa).

Two sets of controls were maintained throughout the experiment. One set was recombinant wings with the right wing bud ectoderm replaced in normal dorso-ventral orientation on the limb mesoderm. The other group of controls were isolated right wing buds or presumptive wing buds. Both controls were grown as flank grafts and processed in the same manner as experimental wings.

Results

Ectodermal Effect on Feather Pattern

The effect of reversing the ectoderm along the dorso-ventral axis is grossly observed on the fully developed wing by a change in the feather pattern. The normal chick wing has a more dense feather

pattern on the dorsal region compared with its ventral region. Along the postero-dorsal margin of the wing is a row of long, primary flight feathers which extend from the elbow to the tip of the autopod. In experimental wings with dorso-ventrally reoriented ectoderm supernumerary feathers occur ventrally, resulting in a bidorsal feather pattern (Fig. I-1^{*}). The bidorsality is more evident at the posterior border of the wing where a double row of primary flight feathers is present.

The extent of the ectodermal influence on the feather pattern of the wing after 180° reorientation of the ectoderm is summarized in Table I-1. Supernumerary feathers are consistently present in the ventral region of composite wings recombined as early as stage 15. At this stage, extra feathers occur ventrally along the whole length of the autopod and in some cases extend to the ventral region of the zeugopod. When the donor embryos were younger than stage 15, the feather pattern is generally normal and corresponds with the dorso-ventral polarity of the mesoderm, regardless of the orientation of the limb ectoderm. Recombinations with stages older than stage 15 generally develop into wings with a consistent bidorsal feather pattern at the autopod. At these older stages, regions proximal to the autopod have extra feathers ventrally. For instance, a stage 16 recombinant wing has a posterior double row of long flight feathers extending from the elbow to the tip of the autopod.

* All tables and figures may be found in the appendix at the end of the chapter.

The ectodermal influence on the feather pattern seems to be most extensive at stage 17-18. Ventral supernumerary feathers are found at the stylopod and distal regions (zeugopod and autopod) are bidorsal. At stage 19-21, there appears to be a loss of ectodermal effect at the proximal levels of the wing. The stylopod of recombinant wings has the dorso-ventral polarity of the mesoderm (normal feather pattern) while extra feathers occur ventrally at more distal regions. The proximal limit ventrally of extra feathers is mid-zeugopodium for stage 19-20 and wrist level for stage 21. Although the general feather pattern in experimental wings is bidorsal, complete dorso-ventral reversals occur at digit 2 and in some cases at the distal half of the autopod at stages which showed the ectodermal effect.

Ectodermal Effect on Muscle Pattern

Experimental wings with ventral, supernumerary feathers were serially sectioned and stained to analyze the muscle and cartilage patterns. The evaluation of the ectodermal influence on muscles and skeletal elements is limited to the autopod, the region of the wing with consistent feather-pattern alteration after reversal of the limb ectoderm. In normal wings, an asymmetric distribution of muscles exists in the autopod. Ten muscles are present, four extensors dorsally and six flexors ventrally (Table I-2).

Experimental wings from stage-14 donor embryos develop normal muscle pattern corresponding to the dorso-ventral polarity of the mesoderm after dorso-ventral inversion of the ectoderm (Table I-2). This agrees with the earlier observation on the feather pattern which

is usually normal, also conforming with the polarity of the mesoderm. The earliest effect of the ectoderm on muscle pattern occurs at stage 15. After dorso-ventral reversal of the ectoderm at stage 15, half of the wings sectioned have a dorso-ventral muscle pattern corresponding to the polarity of the ectoderm rather than the mesoderm. However, unlike the feather pattern alteration which is incomplete, i.e., bidorsal, the muscle pattern reversal is nearly complete. The remainder of the sectioned stage 15 experimental wings have a normal muscle pattern conforming with the mesoderm, despite having a bidorsal feather pattern.

Nearly all recombinant wings from stage 16-21 embryos display the normal asymmetry of the muscles of the autopod but in dorso-ventrally reversed arrangement, i.e., corresponding to the reversed ectoderm. The typical muscle pattern of experimental wings is shown in Fig. I-2 approximately at the same levels as in the control wings. Seven of the ten muscles in the autopod are frequently reversed (Table I-2). The development of these muscles is variable. They are usually smaller than normal and in some cases absent, particularly those associated with digit 2 (AbI and FI) and digit 4 (FDQ). Some muscles show partial reversal and have bidorsal symmetry, usually the interosseus (IOD) and ulnometacarpalis dorsalis (UMD) muscles. The bidorsal muscles exist alongside those muscles which are dorso-ventrally reoriented in the same wing (Fig. I-3). Of the two muscles of the autopod which tend to be bidorsal, the latter (UMD) is the most proximal and shows the least tendency to be reversed in experimental wings.

Tendons associated with some of the autopod muscles also show a 180° reorientation after dorso-ventral reversal of the limb ectoderm. For example, the abductor medius AbM), found in control wings ventral to the third metacarpal is always accompanied by a large tendon (Fig. I-4a) which in experimental wings appears dorsal to the third metacarpal associated with the reversed AbM (Fig. I-4b). The other tendons in the autopod although well-developed and prominent in controls, tend to be small in recombinant wings often merging with the perichondrium of the cartilage or absent.

Ectodermal Effect on Skeletal Pattern

The dorso-ventral reversal of the limb ectoderm also results in changes in shape and orientation of the skeletal elements of the autopod. In cross-section, the proximal phalanx of the third digit in normal wings has a triangular shape with its tapered end oriented ventrally and posteriorly (Fig. I-4c). In contrast, the same cartilage in experimental wings while retaining its triangular shape orients dorsally and posteriorly (Fig. I-4d). In some instances, this cartilage shows a partial shift in position such that the tapered end points midposteriorly.

At more proximal levels (cross-section through digits 2-4), the third metacarpal has a projection at its posterodorsal border (Fig. I-2a). Experimental wings also have this protruberance that extends from the postero-ventral region of the third metacarpal (Fig. I-2b). Some wings have two projections which occur as postero-dorsal and postero-ventral extensions from the third metacarpal, a result that

may indicate a bidorsal cartilage pattern. At this level, the fourth metacarpal is slightly dorsal relative to the third metacarpal as opposed to a slightly ventral position in controls. In some wings, the change in orientation is not complete, and the fourth metacarpal is medial and posterior to the third digit.

At the wrist level, a third marker which indicates ectodermal influence is the position of the fifth metacarpal which normally appears posterior and ventral to the fourth metacarpal (Fig. I-2c). In comparison, the fifth metacarpal of recombinant wings has a postero-dorsal or mid-posterior orientation relative to the fourth metacarpal (Fig. I-2d). In general, the ectodermal effect on cartilage pattern is more variable compared with the muscle pattern. At the same stage of development both complete and partial reversals in cartilage orientation are observed.

Discussion

Dorso-ventral differentials in the developing chick limb can be recognized at the limb-bud stage by a more rounded dorsal side and a rather flat ventral side. The dorso-ventral reorientation of the limb ectoderm alters this shape such that the dorsal mesoderm flattens and the ventral side becomes more rounded. This change in shape can be recognized in composite wing buds sixteen hours after transplantation to host embryos (Geduspan and MacCabe, 1986). The results reported here show that subsequently, when fully developed, the wings have dorso-ventrally reversed structures. The ectodermal effect is seen in

both epidermal and mesodermal derivatives. The reversal of epidermal structures is incomplete, generally in the form of a bidorsal feather pattern on fully developed wings. Results of similar experiments with the leg show a more complete dorso-ventral reversal of epidermal structures (MacCabe et al., 1973; Patou, 1977). The influence of the reoriented ectoderm, however, is not confined to the integumentary derivatives. In our studies, the effect extends to the muscle and cartilage patterns which are reversed more completely than the feather pattern. The majority of the muscles of the autopod retain their asymmetry but in dorso-ventrally reversed arrangement. In addition, skeletal elements with recognizable dorso-ventral asymmetry are completely or partially reversed.

That the ectoderm can influence the dorso-ventral skeletal pattern is not unexpected. MacCabe et al. (1973), after similar experiments with the leg, suggested the skeletal elements are affected based on the curvature of the toes and flexion of the tibiotarsal joint. Searls arrived at the same conclusion when skeletal abnormalities develop as a result of reorienting blocks of limb ectoderm and subjacent mesoderm (Stark and Searls, 1974; Searls, 1976). In vitro studies have implied an ectodermal effect on limb mesoderm differentiation. Removal of the ectoderm from the limb-bud explants causes the mesoderm to differentiate as cartilage rather than muscle (Kosher et al., 1979; Searls and Smith, 1982). Conversely, limb ectoderm can inhibit chondrogenesis of limb mesenchyme in culture (Solursh et al., 1981; Solursh, 1984b). Our results thus extend these studies by showing that in vivo reorientation of the ectoderm along

the dorso-ventral axis reverses the dorso-ventral pattern of the skeletal elements of the autopod. It is clear from these results that the ectoderm does more than simply insure chondrogenesis at the core of the limb (Searls and Smith, 1982; Kosher et al., 1979); also can control the details of the dorso-ventral pattern of cartilages and muscles. Differences that exist between dorsal and ventral ectoderm may specify the details of the pattern (Millaire, 1965).

The most significant effect of the dorso-ventrally reversed ectoderm is on the muscle pattern of the autopod. The normal muscle pattern of the limb arises by progressive subdivision of the dorsal and ventral premuscle masses (McLachlan and Wolpert, 1982) which in the autopod region form four extensors dorsally and six flexors ventrally. Our results show that the dorso-ventral reorientation of the limb ectoderm has altered the muscle pattern such that flexor muscles of the autopod occur dorsally and the extensor muscles ventrally. The limb ectoderm could affect any of the phases of muscle morphogenesis to bring about a change in the muscle pattern. It is unlikely, however, that the migration of the premyogenic cells into the distal region of the limb is affected even though many of our recombinations were done at stages before the presumptive muscles reach the prospective autopod region (Newman et al., 1981). Previous studies have shown that the migration of presumptive myoblasts into the limb is not region-specific (Mauger and Kieny, 1980), and premyogenic cells are distributed at random on individual muscles (Kieny and Chevalier, 1980). What appears to be altered is the individuation of the different muscles in the autopod. In response to

an ectodermal cue, the premuscle mass in the dorsal region of experimental wings may cleave to form at least four of the six flexor muscles and in the ventral region gives rise to at least three of the four extensors. The relative number of dorsal and ventral muscles in the autopod thus conforms to the ectodermal signal. In both control and experimental wings, more muscles are associated with the ventral than dorsal ectoderm. In addition, the positions of the origins and insertions are affected by the ectoderm resulting in a relatively normal spatial relationship between reversed muscles and reversed skeletal elements.

The mechanism by which the ectoderm can influence cartilage and muscle patterns is not known. It is possible that the limb ectoderm may have changed the extracellular environment surrounding the differentiating mesodermal derivatives. Earlier studies have shown that connective tissues have morphogenetic influence on the muscle pattern of the limb (Jacob and Christ, 1980; Chevalier and Kieny, 1982). Therefore changes in the extracellular matrix in terms of the distribution of extracellular materials (Singley and Solursh, 1981) or as a result of mechanical traction exerted by migrating mesenchyme cells (Harris et al., 1981) could alter the spatial arrangement of differentiating cells. That the ectoderm does affect the extracellular matrix has been suggested by in vivo (Solursh et al., 1979; Lunt and Seegmiller, 1980; Kosher and Savage, 1981) and in vitro (Solursh et al., 1984; Zanetti and Solursh, 1984; Zanetti and Solursh, 1986) experiments. In vivo, secretion of hyaluronic acid and other glycosaminoglycans by the ectoderm from young embryos (Solursh et al.,

1979) or from limb buds (Kosher and Savage, 1981a) appears to affect the morphogenesis of the adjacent mesodermal tissue. The turnover of these extracellular materials during limb development is correlated with the onset of differentiation in the limb mesoderm (Singley and Solursh, 1981; Kosher and Savage, 1981b; Melnick et al., 1981; Kosher et al., 1982). In vitro, the extracellular matrix has been demonstrated to effect migration of myoblasts (Turner et al., 1983; Solursh, 1984c) as well as mesodermal differentiation (Solursh et al., 1984; Swalla and Solursh, 1984; Zanetti and Solursh, 1984). The epithelial effect in culture is mediated by secretion from the ectoderm which modifies the extracellular matrix (Zanetti and Solursh, 1984) or by affecting the level of extracellular materials deposited by the adjoining mesenchyme cells (Merrilees and Scott, 1980).

Our results do not suggest that the mesoderm has no role in the control of dorso-ventral polarity in the chick. This study confirms the results of previous studies that before limb outgrowth begins, the control of dorso-ventral polarity resides in the mesoderm. Ectoderm reoriented at stage 14 does not alter the dorso-ventral polarity of the wing, i. e., the polarity conforms with the mesoderm. Our results suggest that a transition occurs at stage 15 wherein the control of dorso-ventral polarity shifts to the ectoderm. At stage 15, about half of the wings show dorso-ventral reversal, whereas at stage 16 all of the experimental limbs have their distal dorso-ventral polarity conforming with the reversed ectoderm. Earlier experiments, however, have shown mesodermal control as late as stage 17 (Kieny, 1960; Dhouailly and Kieny, 1972; Saunders and Reuss, 1974). These earlier

experiments involved limb mesoderm transplanted to host embryos and allowing the host ectoderm to heal over the transplant. Under these conditions we also observed dorso-ventral control by the mesoderm (results not shown) as late as stage 17. Healing ectoderm apparently has some unique properties. For example, it responds to ridge induction by the underlying mesoderm under conditions where non-proliferating ectoderm cannot (Saunders and Reuss, 1974. Different techniques of recombining tissues thus yield a slightly different timing of tissue interaction. Nevertheless the results show clearly that at about the time of onset of limb outgrowth, some of the control of dorso-ventral spatial patterns shifts from the mesoderm to the ectoderm. The dorso-ventral information acquired by the ectoderm comes from its association with the presumptive limb mesoderm (work in progress). This acquisition of dorso-ventral information may not be unique to the limb-forming region. Back ectoderm in combination with limb mesoderm results in the formation of a bidorsal limb (Errick and Saunders, 1976), and ventral flank ectoderm in association with dorsal limb mesoderm results in the formation of biventral digits (Carrington and Fallon, 1986). These results also suggest that the ectoderm's dorso-ventral information is rather non-specific, the specificity resides in the mesodermal response.

Several models have been proposed to account for pattern formation in the limb based on the results of transplantation experiments that rearrange the components of the limb (French et al., 1976; Bryant et al., 1981; Iten, 1982; Javois, 1984; Newman and Frisch, 1979; Tickle et al., 1975; Wolpert, 1971). Of these models

only the polar- coordinate model attempts to account for specification of the dorso-ventral pattern, patterning along the three limb axes is accounted for by a common mechanism, intercalation, which experiments have shown to resolve pattern discontinuity by insertion of missing positional values. However positional information according to this model is in the limb mesoderm, the overlying ectoderm having none (French et al., 1976; Bryant and Iten, 1976; Javois, 1984). Without modification, this model is inconsistent with our results which show that the ectoderm can significantly affect the dorso-ventral pattern once limb outgrowth begins. Since the polar coordinate model fits the developing and regenerating amphibian limb better than the developing chick limb (Bryant and Muneoka, 1986), our results may suggest a difference between chickens and amphibians rather than between limb development and regeneration as suggested by Tickle (1981).

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PART I
APPENDIX

Table I-1. Feather pattern of recombinant wings after dorso-ventral reversal of the limb ectoderm.

Stage of Ectoderm Reorientation	No. of Wings	EXTENT OF FEATHER PATTERN REVERSAL IN RECOMBINANT WINGS		
		Stylopod +Zeugopod +Autopod	Zeugopod +Autopod	Autopod
14	16	0	1	3
15	25	0	6	20
16	21	2	20	21
17	16	12	16	16
18	31	15	31	31
19	40	1	40	40
20	16	0	16	16
21	22	0	22	22
Control: Recombined Wing	34	0	0	0
Normal Wing	20	0	0	0

TABLE I-2. Number of wings with normal and dorso-ventrally reversed muscles in the autopod after reorientation of the ectoderm.

Stage of Development	14	15	16	17	18	19	20	21	CONTROL ^d Recombinant Wing	Normal Wing
Number of Wings Sectioned	4	4	5	4	5	4	5	4	7	4
Muscles of the Autopod Dorsal Muscles										
EIB	3 ^b	2	2	4	3	4	5	1	2	3
D-V Reversed		2	2	4	3	4	5	1	6	3
Normal	3	2	5(1) ^c	3	5	4	3	1	6	3
D-V Reversed	4	2	5(2)	4(1)	5(2)	1	4(1)	2	6	4
Normal	2	2	1	1	2	2	1	1	6	3
D-V Reversed	4	2	3(1)	3(1)	3(1)	3(1)	3(2)	3(1)	6	3
Normal		2(1)								
D-V Reversed										
Ventral Abi Muscles										
Abi	2	2	2	2	2	3	2	3	3	3
D-V Reversed		2	2	2	2	3	2	3	4	4
Normal	3	2	2	2	1	3	2	3	5	4
D-V Reversed	4	2	3	4	5	4	5	4	7	4
Normal	4	2	4	4	5	4	5	4	6	4
D-V Reversed	4	2	3	3	2	2	4	3	4	4
Normal	4	2	2	2	2	2	2	2	4	4
D-V Reversed		1	3	3	2	2	4	3	4	4
Normal	4	2	2	3	2	2	2	-	4	4
D-V Reversed		1	2	3	2	2	2	-	4	4

- a. Names of muscles: EIB-extensor indicis brevis; EMB-extensor medius brevis; IOD-interosseus dorsalis; UMD-ulnometacarpalis dorsalis; Abi-abductor indicis; FI-flexor indicis; AdI-adductor indicis; AbM-abductor medius; IOP-interosseus palmaris; FDQ-flexor digiti quarti
- b. Indicates missing muscle if the number does not correspond with the number of wings sectioned.
- c. Number in parenthesis indicates bidorsal muscle.
- d. Recombined controls represent stage 16-21; Normal wing controls are from stage 18-20 embryos.

Fig. I-1. Feather follicle distribution on normal dorsal (a) and ventral (b) wing surfaces. In the experimental wing, the ectoderm was dorso-ventrally at stage 16 resulting in a dorsal pattern on both sides (c. dorsal and d. ventral sides relative to the mesoderm) except for digit 2 (arrows) which has a dorso-ventral pattern conforming with the ectoderm.

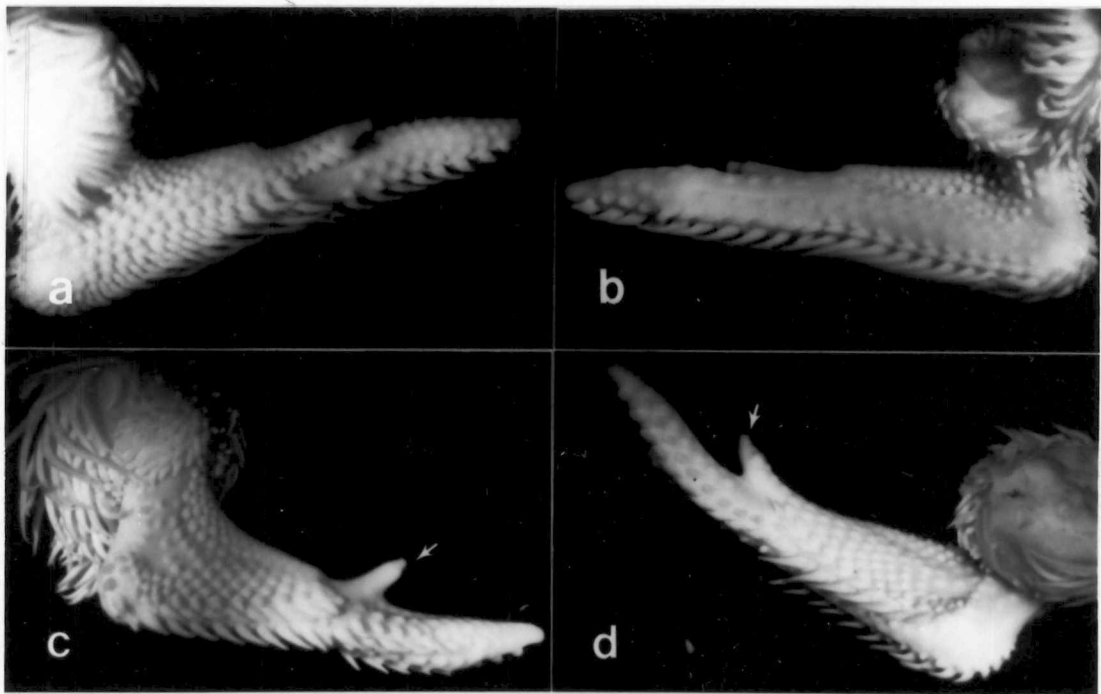


Fig. I-2. Cross-sections of a normal wing and an experimental wing after the ectoderm was reoriented at stage 18. Sections of control (a) and experimental (b) wings at the level of digits 2, 3, and 4, showing the postero-dorsal projection (arrow) of the third metacarpal in (a) which occurs in reversed position in (b). In addition, the position of dorsal and ventral muscles are reversed (see Table I-2 for abbreviations of muscles). Also at the wrist level (c. control; d. experimental), the muscles of the recombinant wings are reversed along with the spatial orientation of the skeletal elements. Note also the position of the fifth metacarpal (5). Orientation relative to the mesoderm of recombinant limb bud: dorsal, top; anterior, right; ventral, bottom; posterior, left.

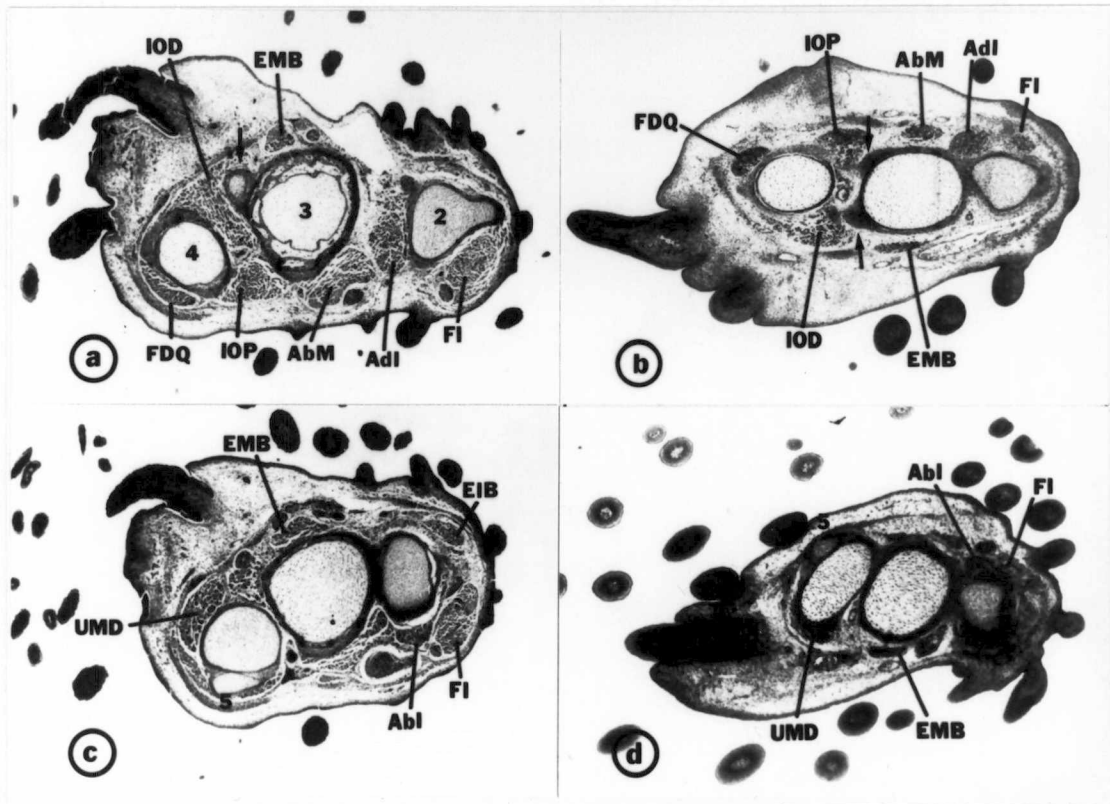


Fig. I-3. (a) Section through the midautopodium of a wing after ectodermal reorientation at stage 15 showing the bidorsal muscle interosseus dorsalis (IOD) alongside dorso-ventrally reversed abductor medius (AbM). (b) Proximal autopodium of an experimental wing (after dorso-ventral reversal of limb ectoderm at stage 16) with bidorsal ulnometacarpalis dorsalis (UMD) and dorso-ventrally reversed flexor indicis (FI). Orientation relative to the mesoderm of recombinant limb bud: dorsal, top; anterior, right.

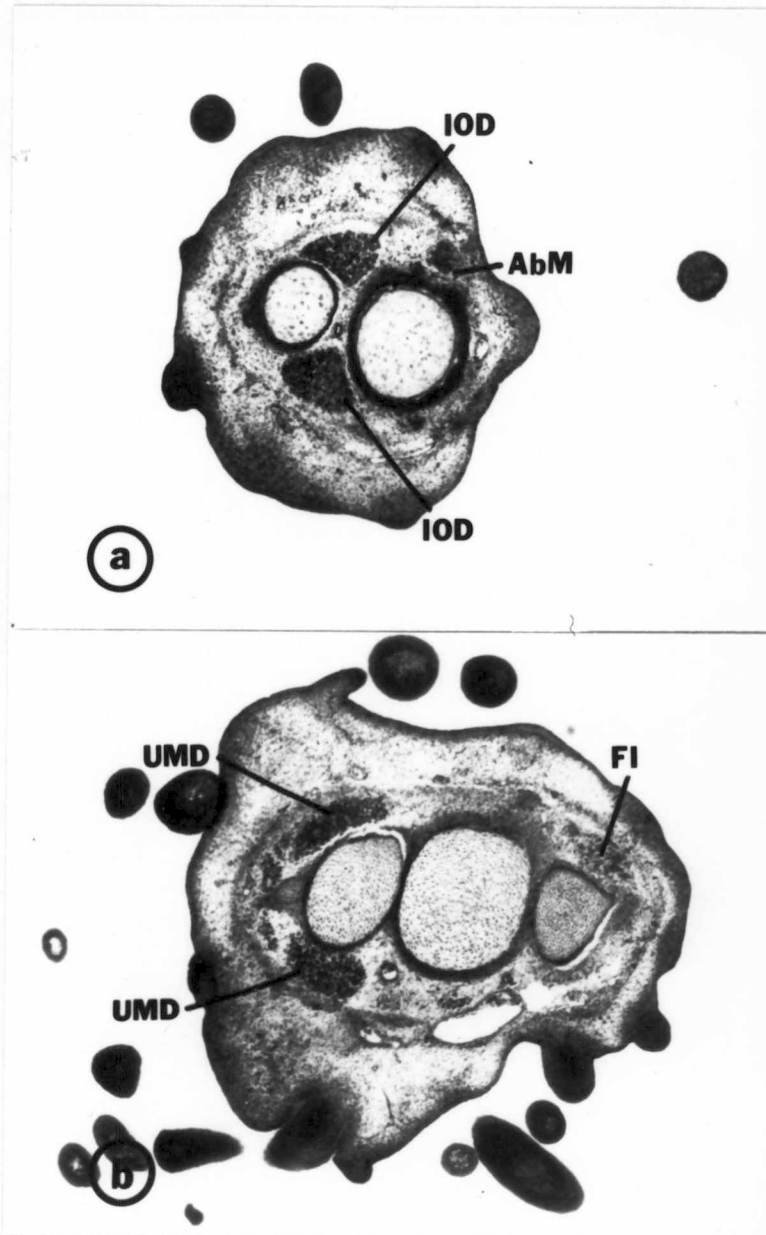
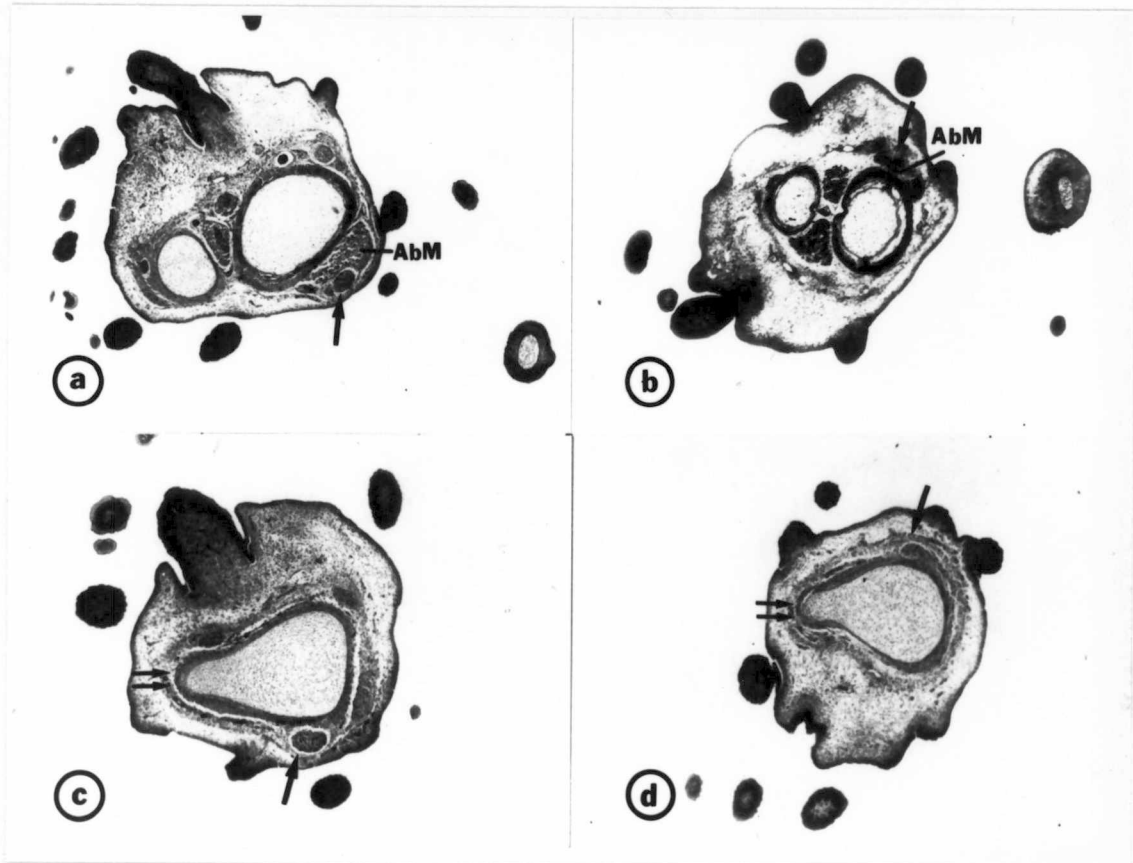


Fig. I-4. Sections of control and experimental (with limb ectoderm dorso-ventrally reoriented at stage 20) wings showing distal muscle and skeletal patterns in the autopod: (a) and (b) are sections through the midautopodium of control and experimental wings respectively. The abductor medius (AbM) is accompanied by a large tendon (arrow) of a muscle in the zeugopod. Distal sections of the same wings show the proximal phalanx of digit 3. Note the reversed orientation of the phalanx as seen by the posterior projection (double arrows) in experimental wing (d) compared with the control (c). The tendon associated proximally with the AbM muscle is shown ventral to the phalanx in control (arrow) and dorsal in the experimental wing. Orientation relative to the mesoderm of the recombinant limb bud: dorsal, top; anterior, right.



PART II

TRANSFER OF DORSO-VENTRAL INFORMATION FROM MESODERM TO
ECTODERM AT THE ONSET OF LIMB DEVELOPMENT

Introduction

Normal development of the vertebrate limb involves a series of interactions between mesoderm and ectoderm which promotes outgrowth and specifies the spatial organization of structures in the limb. For instance, important interactions exist between limb mesoderm and ectoderm during the establishment of the proximo-distal axis. The early limb mesoderm induces the formation of the apical ectodermal ridge (Kieny, 1960; Saunders and Reuss, 1974). The ectodermal ridge, in turn, promotes outgrowth of the underlying limb mesoderm and elaboration of distal structures (Saunders, 1948; Summerbell, 1974). The control of proximo-distal patterns of differentiation, however, resides in the mesoderm (Rubin and Saunders, 1972). The limb mesoderm also serves to maintain the ridge in its active, thickened form (Zwilling and Hansborough, 1956; Saunders and Gasseling, 1963; Zwilling, 1974), and control the spatial organization of structures along the antero-posterior axis (Zwilling, 1956; Hinchliffe and Gumpel-Pinot, 1981; Summerbell and Honig, 1982).

The relative roles of the limb mesoderm and ectoderm in the control of dorso-ventral patterns, however, are more complex. Transplanting prospective limb mesoderms in various dorso-ventral orientations to the flank of host embryos in combination with healing host flank ectoderm result in wings with their dorso-ventral polarity conforming to the mesodermal orientation (Saunders and Reuss, 1974). Other studies, however, show that when intact limb ectoderm is dorso-ventrally reversed relative to the mesoderm an ectodermal effect

is observed on the distal region of the resulting limb evaluated by the distribution of epidermal derivatives (MacCabe et al., 1974; Patou, 1977). We have recently shown that this ectodermal influence extends to mesodermal structures as well, affecting muscle and cartilage patterns in the autopod (Geduspan and MacCabe, 1986 and in preparation). The ectodermal effect is observed as early as stage 15. At stage 14 and younger, control of dorso-ventral pattern resides in the limb mesoderm. Thus at stage 15 either the ectoderm acquires dorso-ventral information or the mesoderm becomes capable of responding to an existing ectodermal signal. These questions are addressed in this investigation. In addition, we also demonstrate that the ectoderm does not obtain its dorso-ventral information autonomously but gets this information from the mesoderm.

Materials and Methods

The experiments were performed on White Leghorn chick embryos. The fertile eggs were obtained from the Department of Animal Science, University of Tennessee, Knoxville. After two to three days of incubation in a humidified incubator at 38°C, 2 ml albumin was removed from each egg and windows cut in the shell. The windows were resealed with Parafilm (American Can Co., Connecticut) and the eggs returned to the incubator until the appropriate stages were obtained. The following experimental manipulations were done with the embryos.

The first series of experiments were recombinations of stage 14-16 mesoderm in association with dorso-ventrally reversed limb

ectoderm from younger, same stage, or older embryos. Right presumptive wings were excised and transferred to cold phosphate-buffered saline (PBS). After rinsing in calcium-magnesium free (CMF) PBS, they were incubated at 38°C for 20 minutes in 1% EDTA in CMF-PBS. After removal of their overlying ectoderm, the presumptive limb mesoderms were returned to cold PBS until the ectoderm donors were ready.

The limb ectoderm came from stage 14-18 left prospective wing buds. The ectoderm donors were incubated in 1% trypsin in CMF-PBS for 1 1/2-2 hours at 4°C, after which they were transferred to cold 2:1 mixture of Tyrode's- Horse serum solution. The ectoderm was separated from its underlying mesoderm and immediately recombined with stage 14-16 right presumptive wing-bud mesoderm in dorso-ventrally reversed orientation. The recombinants were transferred to another solution of Tyrode's-Horse serum and allowed to warm up to room temperature. The composite presumptive limbs were grafted to stage 19-21 host embryos and allowed to develop for an additional ten days. Controls for these experiments were excised presumptive right wings or recombined wings with the ectoderm replaced in normal dorso-ventral orientation on the limb mesoderm.

In the second series of experiments, ectoderm was dorso-ventrally reversed on the mesoderm for 16 hours and then reisolated and combined with limb mesoderm in normal dorso-ventral orientation. For these "double" recombinations presumptive limb mesoderm and ectoderm were isolated using the procedure described in the preceeding paragraphs. Stages 14 and 16 left presumptive wing mesoderm were initially recombined with stage 16 right presumptive wing bud ectoderm in

dorso-ventrally reversed orientation. The recombinants were transplanted to the flank of stage 19-21 host embryos and incubated for 16 hours, after which, the grafts were excised and the limb ectoderm was reisolated using the procedure described previously. The recovered ectoderm was immediately replaced on a stage 19 limb mesoderm in its normal dorso-ventral orientation. The composite limb buds were grafted to stage 19-21 host embryos and incubated for another ten days.

Host embryos were sacrificed on the tenth day of incubation. The fully-developed wings recovered were analyzed for ectodermal effects by examination of the feather follicle pattern. The wings were fixed in Bouin's fixative. At least four wings from each recombination were paraffin embedded, serially sectioned at 7 μ m, and stained with Milligan's Trichome stain to study muscle and cartilage patterns at the autopod. Muscles were identified according to their origin and insertion (Sullivan, 1962; Shellswell, 1977).

Results

Presumptive limb mesoderm from stage 14-16 embryos were recombined with the same stage prospective limb ectoderm in dorso-ventrally reversed orientation and allowed to develop as flank grafts. The results are shown in Table II-1, experiment a. Thirteen of the sixteen wings recovered from stage 14 donors have a feather pattern conforming to the polarity of the mesoderm. Histological examination of the muscle and cartilage patterns of at least four of

these recombinant wings from each stage show that experimental wings from stage 14 donors have muscle and cartilage patterns conforming with the mesoderm (Figs. II-1c, d). This agrees with our initial observations on feather-pattern alteration and confirms the results of previous studies (Geduspan and MacCabe, in preparation; Kieny, 1968; Saunders and Reuss, 1974) suggesting that dorso-ventral polarity at this stage is under mesodermal control. On the other hand, wings obtained from stage-16 donors have a bidorsal feather pattern at their distal regions, a result indicating an ectodermal influence, while the proximal regions have the mesodermal pattern. The muscle and cartilage patterns of sectioned recombined wings from stage 16 donors show an almost complete dorso-ventral reversal at the autopod, i.e., the pattern conforms to the polarity of the ectoderm (Figs. II-1e, f). These results suggest that a transition occurs during stage 15 which allows limb ectoderm to control the dorso-ventral polarity of the distal region of the wing.

The failure of stage 14 dorso-ventrally reversed ectoderm to influence the differentiation of the limb mesoderm suggests three possibilities. First, there may be an absence of dorso-ventral information in the ectoderm of prelimb stages, the mesoderm may be unable to respond to an ectodermal signal, or both may occur. To distinguish between these alternatives, the recombinations were done with donors from different stages. Stage 14 presumptive limb ectoderm was isolated and replaced in dorso-ventrally reversed orientation on a stage 16 presumptive limb mesoderm. This mesoderm, according to the above results, is capable of responding to an ectodermal influence.

The results are shown in Table II-1, experiment b. The recombinant wings, in general, have a bidorsal feather pattern at the autopod. However, the four wings sectioned have normal muscle and cartilage patterns, conforming with the mesoderm (Figs. II-2a, b). These findings suggest that stage 14 ectoderm lacks dorso-ventral information capable of influencing the pattern of differentiation of muscles and cartilages in the autopod.

Can an older ectoderm, which already has its dorso-ventral information, then influence the differentiation of a young mesoderm? To answer this question, stage 14 presumptive limb mesoderm was recombined with dorso-ventrally reversed ectoderm from stage 15-18 embryos, ectoderms which are capable of influencing mesodermal differentiation (Geduspan and MacCabe, in preparation). In combination with dorso-ventrally reversed stage 15-16 ectoderm, stage 14 mesoderm developed into wings with normal feather patterns (Table II-1, experiment c). Experimental wings (recombination of stage 14 mesoderm and stage 16 dorso-ventrally reversed ectoderm) have normal muscle and cartilage patterns. However, in recombinations using limb ectoderm from stage 17-18 donors more wings show feather pattern alteration usually a bidorsal or biventral pattern at the autopod. One of the wings sectioned from recombinations with stage 17 as ectoderm donor has a single biventral muscle; the rest have normal polarity conforming to the mesoderm. The cartilage pattern also conforms to the polarity of the mesoderm. With wings derived from recombinations using stage 18 ectoderm, three of the four wings sectioned have changes in muscle pattern. Some muscles appear biventral while others may be

dorso-ventrally reversed or normal (conforming with the mesoderm). The ectodermal effect on muscle pattern is minimal and involves only a few muscles in the autopod (Figs. II-2e, f). Three of the experimental wings sectioned have skeletal pattern reversal. One of the wings has a missing digit 4 and fifth metacarpal, therefore analysis of skeletal pattern proved difficult. The results show that, stage 14 mesoderm is generally incapable of responding to the dorso-ventral message from stage 15-17 ectoderms, although there is some response if the ectoderm is from a stage 18 donor.

Mesoderm older than stage 14, however, has increased ability to respond to the dorso-ventral message from the ectoderm. When stage 15 presumptive limb mesoderm was recombined with stage 16 limb ectoderm in dorso-ventrally reversed orientation, most of the wings recovered have supernumerary feathers on their ventral regions (Table II-1, experiment d). Three of the five wings sectioned have one or two muscles at the autopod showing a biventral or bidorsal pattern. Accompanying the muscle pattern changes is also a reversal of cartilage orientation usually involving the third phalanx of digit 3. This ectodermal effect is further enhanced using stage 17 ectoderm in the recombination experiments. Most of the experimental wings have a bidorsal feather pattern and in a few cases are biventral. Of the four sectioned wings, three have muscle pattern changes involving one to five of the ten muscles in the autopod. The same wings which showed muscle pattern alteration also show changes in skeletal pattern. The results with stage 15 mesoderm are thus intermediate between those obtained with stage 14 and stage 16 mesoderms.

The above results suggest that the ectoderm gains dorso-ventral information between stages 14 and 16, i.e., during stage 15. Does this information come from the mesoderm? To answer this question, a double recombination experiment was done as described in the materials and methods. Stage 16 presumptive limb ectoderm was dorso-ventrally reoriented on stage-14 mesoderm and incubated for sixteen hours, the length of time it takes to observe a complete change in shape of the composite limb bud after dorso-ventral reorientation of the ectoderm (Geduspan and MacCabe, 1986). The ectoderm was then removed and placed on a stage-19 mesoderm in normal orientation. The results are shown in Table II-2. All experimental wings have bidorsal feather patterns at the autopod. The five sectioned wings have dorso-ventrally reversed or bidorsal muscle patterns. The change in the muscle pattern is reflected in the skeletal pattern which is also dorso-ventrally reversed (Figs. II-3a, b). In contrast, experimental wings from similar recombinations with stage 16 instead of stage 14 mesoderm have primarily normal feather patterns and only in two cases have bidorsal feather distribution. Muscle and skeletal patterns of these wings are normal (Figs. II-3c, d). The results show that information transfer from the limb mesoderm to the overlying ectoderm has occurred during the sixteen hour period beginning at stage 14. After stage 16 no detectable information transfer takes place within the same period.

Discussion

Prior to the outgrowth of the limb from the body wall, the control of muscle and skeletal dorso-ventral patterns resides primarily in the mesoderm (Geduspan and MacCabe, in preparation). Reversing the dorso-ventral orientation of the ectoderm at stage 14 does not alter muscle and cartilage patterns, i.e., they conform to the mesodermal polarity. A change occurs between stage 14 and 16, however. By stage 16, limb ectoderm when replaced in dorso-ventrally reversed orientation on stage 16 mesoderm can impose its polarity on the mesoderm and reverse the muscle and skeletal patterns in the autopod of resulting wings. The failure of stage 14 ectoderm to reverse the polarity of the underlying stage 14 mesoderm is apparently due in part to the absence of dorso-ventral information in the ectoderm. At this stage (stage 14), limb ectoderms replaced in dorso-ventrally reversed orientation on stage 16 mesoderms form wings with normal (mesodermal) dorso-ventral polarity. From this we conclude that during stage 15 the ectoderm gains dorso-ventral information. It seems likely that this information comes from the mesoderm. This is confirmed by the results of the "double" recombination experiments. After residing reversed on limb mesoderm for 16 hours, beginning at stage 14, the ectoderm when recombined in normal orientation is capable of considerable alteration of mesodermal dorso-ventral differentiation. The mesoderm is thus capable of "programming" the ectoderm with its dorso-ventral information in the period following

stage 14 but not after stage 16. The mesodermal message which programs the ectoderm is therefore a brief one, occurring between stages 14 and 16, a period of about eight to ten hours.

Older ectoderm, which has dorso-ventral information, has limited capacity to change the polarity of a young mesoderm. Recombinations of stage 14 mesoderm with older ectoderm (stages 15-18) in dorso-ventrally reversed orientation gave two sets of results depending on the age of ectoderm donors. Dorso-ventrally reversed ectoderm from stage 15-17 embryos recombined with stage 14 mesoderm can form wings with normal dorso-ventral characteristics including muscle and cartilage patterns. These results again suggest that in the hours following stage 14, the mesoderm can reprogram the ectoderm, even though this ectoderm (stage 15-17) has already been provided with dorso-ventral information. The mesoderm, however, is less able to do this with older ectoderm (stage 18), suggesting some kind of stabilization of the dorso-ventral information in the ectoderm after the initial receipt of the information at stage 14-16. In these recombinations, the resulting wings have biventral muscles. The significance of this biventral pattern is not clear. It may suggest that separate dorsal and ventral messages exist in the mesoderm and the timing of their transfer to the overlying ectoderm differ.

Slightly older limb mesoderm has decreased capacity to reprogram the dorso-ventral information in the limb ectoderm. Stage 15 mesoderm in association with stage 16-17 ectoderm in dorso-ventrally reversed orientation develop into wings with bidorsal feather pattern. These wings have limited reversal of muscle and cartilage patterns in the

autopod. There is a partial ectodermal influence on mesodermal derivatives such that only a few muscles in the limb are reversed.

The results of these recombinations between young mesoderm and older ectoderm suggest that the establishment of dorso-ventral polarity in the ectoderm may be a two-step process. The initial step involves acquisition of dorso-ventral information in the ectoderm, followed by gradual stabilization of the dorso-ventral information in the ectoderm. Stabilized ectodermal information is less likely to be reprogrammed by the mesoderm. Whether this stabilization differs qualitatively or quantitatively from the initial acquisition of dorso-ventral information is not known.

Reciprocal interactions between limb mesoderm and ectoderm as described here for the establishment of dorso-ventral pattern are not unusual in the developing limb. This type of interaction also occurs between limb mesoderm and ectoderm in determining the proximo-distal axis of the limb. Initial induction from the mesoderm promotes the formation of the ectodermal ridge, which then stimulates outgrowth of the underlying mesoderm and development of distal structures (Saunders, 1948). The pattern, however, along this axis is controlled by the mesoderm (Rubin and Saunders, 1972). The brief period of dorso-ventral information transfer is similar to the short period of induction by the ZPA of duplication at the graft site (Saunders and Gasseling, 1963; Smith, 1980) or the period of ectodermal ridge induction in the presumptive wing (Kieny, 1968). Once the ectoderm has acquired dorso-ventral information, it can control the spatial organization of distal muscles and skeletal elements. The

dorso-ventral information transfer to the ectoderm occurs at the same time the ectodermal ridge is induced by the mesoderm (Kieny, 1968; Carrington and Fallon, 1984). The formation of the ridge marks the boundary between dorsal and ventral regions of the limb bud. This spatial and temporal correlation between ridge induction and dorso-ventral information transfer may be significant and needs further investigation.

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PART II
APPENDIX

Table II-1. Results of reorienting ectoderm on older, same stage, and younger limb mesoderm.

Experiment	Recombinations		Total No. of Wings	No. of Wings with Feather Pattern Reversal	No. of Wings Sectioned	No. of Sectioned Wings with	
	Mesoderm Donor	Stage of: Ectoderm Donor				Muscle Reversal	Cartilage Reversal
a	14	14	16	3	4	0/4	0/4
	16	16	21	21	5	5/5	5/5
b	16	14	10	8	4	0/4	0/4
c	14	15	6	1	-	-	-
	16	23	7	5		0/5	0/5
		17	10	6	4	1/4 ¹	0/4
		18	10	8	4	3/4	3/4
d	15	15	25	20	4	2/4	2/4
		16	25	16	5	3/5	3/5
		17	17	14	4	3/4	3/4

¹ One muscle shows biventral pattern

Table II-2. Results of reorienting limb ectoderm on mesoderm for 16 hours.

Stage of Mesoderm Donor	Ectoderm Donor	Total No. of Wings Recovered	No. of Wings with Feather Pattern Reversal	No. of Wings Sectioned	No. of Sectioned Wings with Muscle and Cartilage Reversals
14	16	16	16	5	5/5
16	16	10	2	6	0/6

Fig. II-1. Transverse sections of a normal control wing (a, b) and experimental wings with the ectoderm dorso-ventrally reoriented at stage 14 (c, d) and stage 16 (e, f). Phalanx of the third digit (3) has a triangular shape and normally orients ventrally (double arrows) in (a) and (c) but reverses its orientation in (e). Associated with the ventral region is a large tendon (arrow) which is dorsal in (e). Sections (b), (d), and (f) show the proximal level of the autopod. Note the normal muscle of (b) and (d) and its reversal in (f). Orientation relative to the mesoderm: anterior, right; dorsal, top. Abbreviations: IOD - interosseus dorsalis; EMB - extensor medius brevis; FDQ - flexor digiti quarti; IOP - interosseus palmaris; AbM - abductor medius; AdI - adductor indicis; FI - flexor indicis.

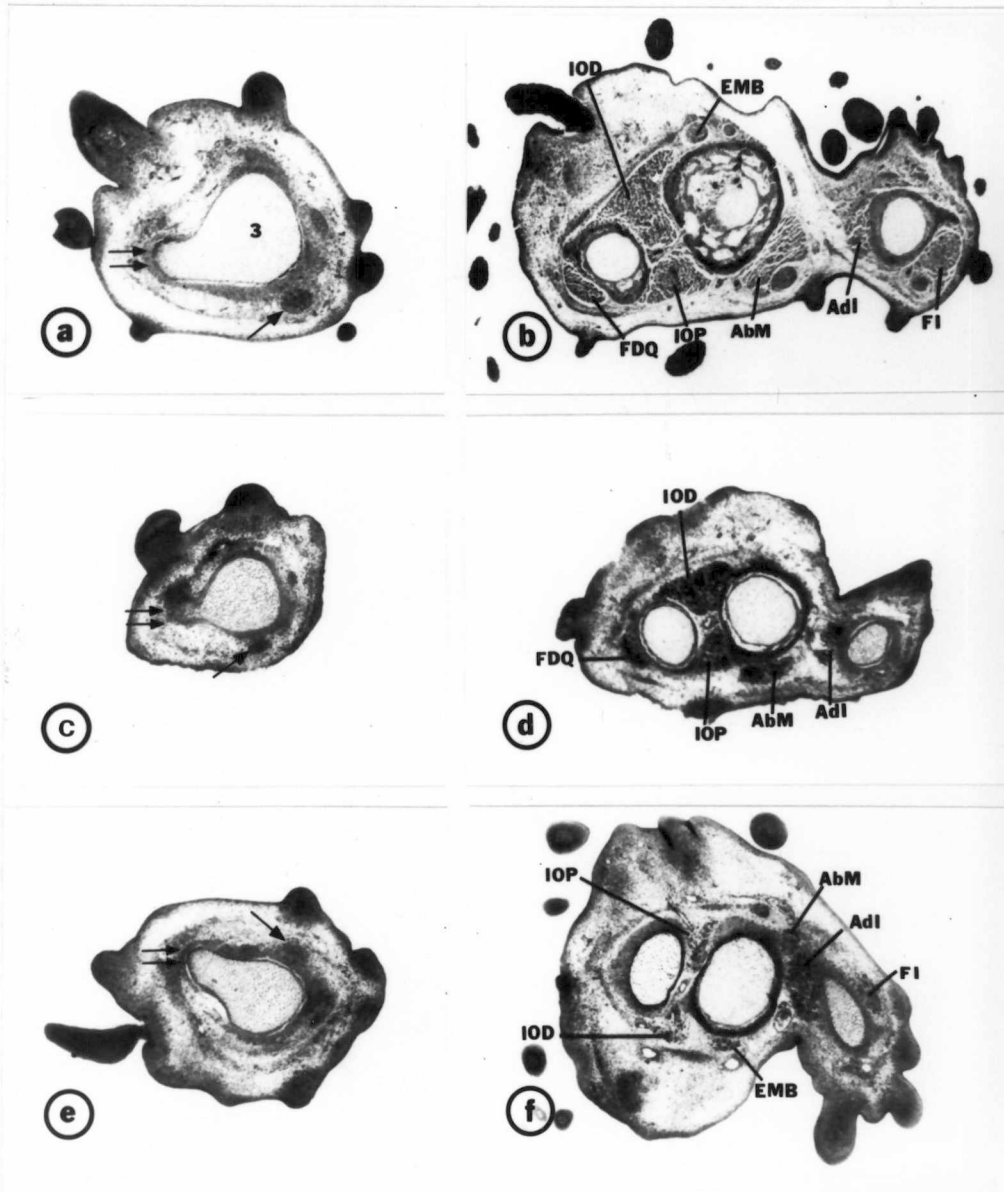


Fig. II-2. Transverse sections of wings resulting from recombinations of stage 14 ectoderm and stage 16 mesoderm (a, b), stage 16 ectoderm and stage 14 mesoderm (c, d). The shape of skeletal elements and distribution of muscles give no indication of a response to ectodermal reorientation (compare with control Figs. II-1a, b). Reorienting stage 18 ectoderm on stage 14 mesoderm, on the other hand, results in partial reversal of muscle and skeletal elements. The shape of the phalanx of digit 3 and the appearance of the two ventral tendons (arrow) suggest a biventral symmetry (e). In a more proximal section (f), one muscle (AbM) is biventral; others are normal or missing. Orientation: anterior, right; dorsal, top.

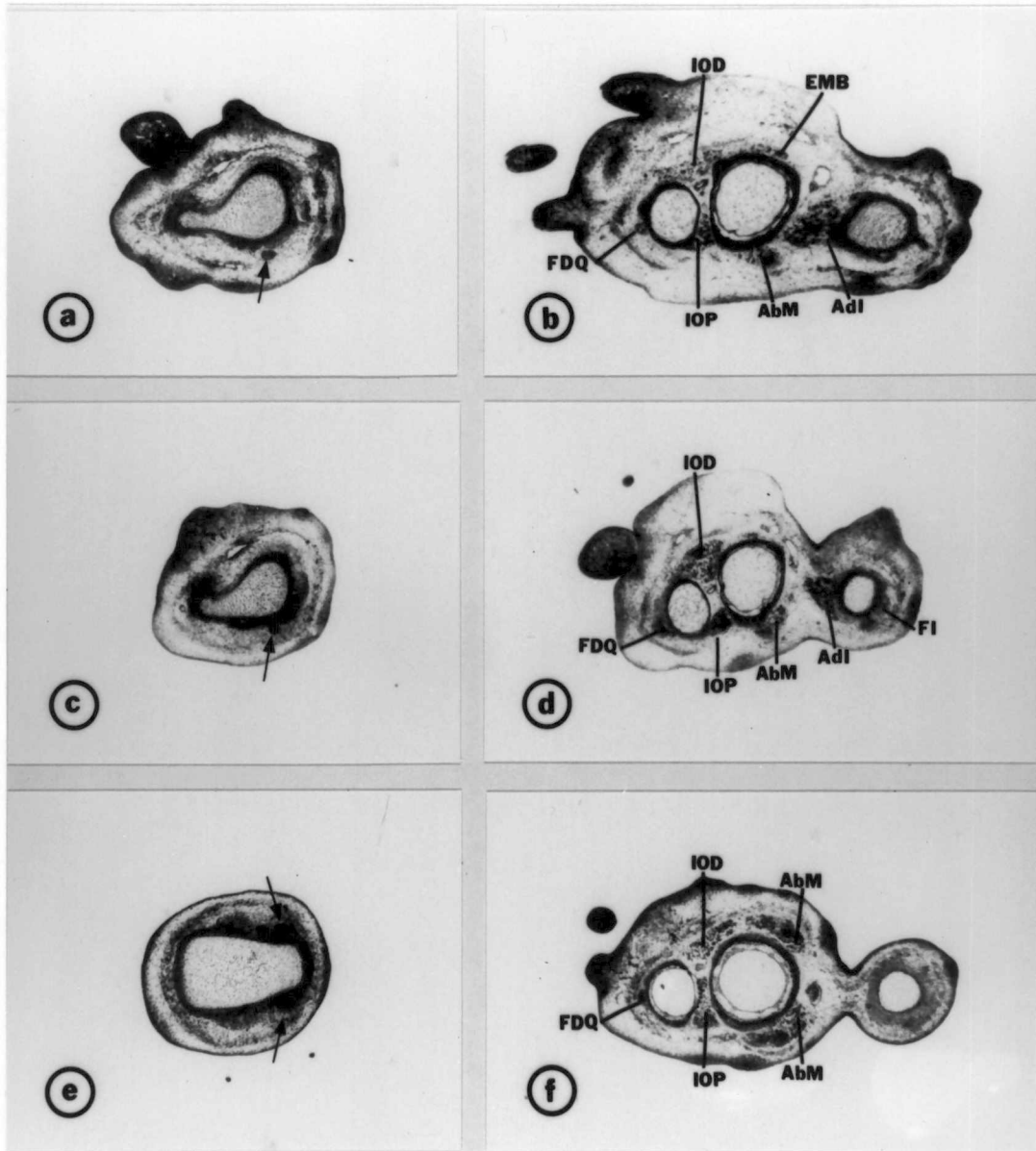
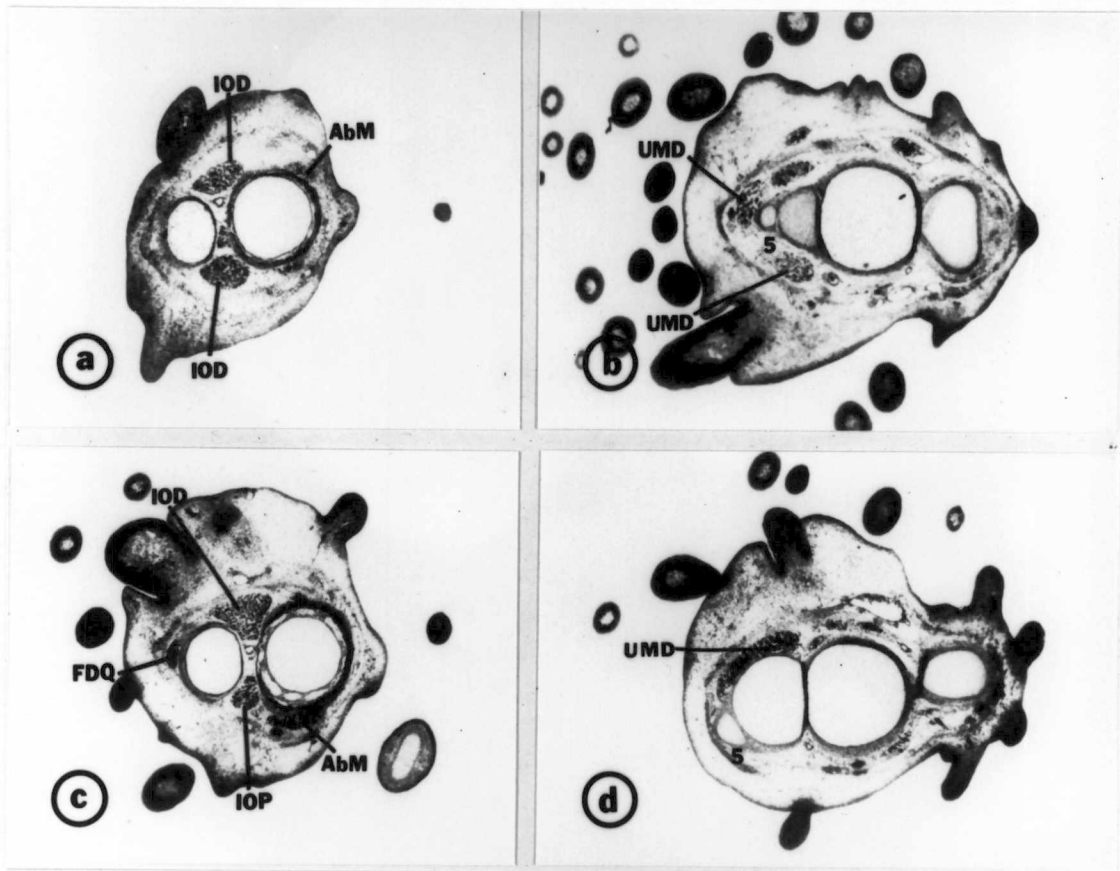


Fig. II-3. Transverse sections of experimental wings resulting from double recombination experiments: stage 16 ectoderm was reversed on stage 14 mesoderm (a, b) and stage 16 mesoderm (c, d) for 16 hours. Note the bidorsal muscles (IOD, UMD) in (a) and (b) and the normal pattern in (c) and (d). The fifth metacarpal (5) has shifted to a midposterior position indicating bidorsal symmetry (d). IOD - interosseus dorsalis; UMD - ulnometacarpalis dorsalis; see Fig. II-1 for abbreviations of other muscles. Orientation: anterior, right; dorsal, top.



PART III

SIGNIFICANCE OF DORSO-VENTRAL DIFFERENTIALS IN THE LIMB IN
RIDGE INDUCTION AND LIMB OUTGROWTH

Introduction

An ectodermal thickening at the distal border of the developing limb, the apical ectodermal ridge, promotes limb outgrowth and elaboration of structures along the proximo-distal axis. The formation of the ectodermal ridge is induced by the underlying limb mesoderm (Kieny, 1968; Saunders and Reuss, 1974; Carrington and Fallon, 1984) which also maintains its thickened configuration (Saunders and Gasseling, 1968; Zwilling and Hansborough, 1956). Promoting limb outgrowth, however, is not the only function of limb ectoderm. The ectoderm peripheral to the apical ectodermal ridge, namely dorsal and ventral ectoderm, influence the development of the distal regions of the limb. Recombination experiments wherein limb ectoderm is dorso-ventrally reversed relative to the mesoderm result in limbs with distal epidermal structures and curvature of the digits conforming to the polarity of the dorso-ventrally reoriented ectoderm (MacCabe et al., 1974; Pautou and Kieny, 1973; Pautou, 1977). The ectoderm can affect not only epidermal derivatives but also muscle and skeletal patterns in the distal region of the limb (Geduspan and MacCabe, in preparation and part I). The dorso-ventral information in the ectoderm comes from the mesoderm which transfers the information to the overlying ectoderm during the early stages of limb development. The ectoderm, once it acquires this information, can determine the dorso-ventral pattern of mesodermal derivatives distally (Geduspan and MacCabe, 1986). The transfer of dorso-ventral information from mesoderm to ectoderm occurs at stages 14-16 (Geduspan and MacCabe, in

preparation and part II), about the same period as the induction of ectodermal ridge by the mesoderm (Kieny, 1968). Morphological dorso-ventral differentials become prominent with the appearance of the ectodermal ridge which marks the border between dorsal and ventral regions of the limb. In the presence of the ridge, a dorso-ventral asymmetry is recognizable in the limb bud by its more rounded dorsal region and flatter ventral side. This study was done to determine whether differences between the dorsal and ventral mesoderm at the onset of limb development are important for the appearance of the apical ectodermal ridge and limb outgrowth.

Dorso-ventral differentials in the presumptive limb mesoderm were eliminated by transplantation of prospective limb (mesoderm plus overlying ectoderm) from a donor embryo to the presumptive limb (without its overlying ectoderm) of the same stage host embryo in dorso-ventrally reversed orientation relative to the host's axes. Under these conditions the ectodermal ridge fails to develop or develops poorly and subsequently no limb outgrowth occurs. We have further characterized the mesodermal dorso-ventral differentials by the capacity of isolated dorsal and ventral halves of the presumptive limb to develop in limb and non-limb (flank) regions.

Materials and Methods

The experimental manipulations were performed on White Leghorn chick embryos. The fertile eggs were supplied by the Department of Animal Science, The University of Tennessee, Knoxville and incubated

in a humidified incubator at 38°C. On the second day of incubation windows were cut in the shell according to standard procedure and resealed with parafilm (American Can Co., Connecticut). The eggs were reincubated until the appropriate stages were obtained.

The first series of experiments were done on stage 14-16 embryos (Hamilton and Hamburger, 1951). Presumptive right and left wings at the level of somites 15-20 were excised and transferred to cold phosphate-buffered saline (PBS). The anterior and dorsal regions of the prospective limbs were marked with carbon in order to determine their orientation during transplantation. The site on the same stage host embryo was prepared by mechanically stripping the overlying ectoderm of the right presumptive leg region. The entire presumptive wing region was transplanted to the right prospective leg area in the following orientation: (a) aadv, dorso-ventral axis of graft and host opposed (b) apdv, both dorso-ventral and antero-posterior axes of graft and host are opposed (c) aadd, both dorso-ventral and antero-posterior axes of graft and host coincide (d) apdd, only the dorso-ventral axis of graft and host coincide. The grafts were tucked at their anterior and posterior ends under the loosened ectoderm of the prospective leg region to hold them in place. The embryos were allowed to warm up to room temperature until the graft adhered to the host tissue and then returned to the incubator.

The grafts were examined 48 hours after transplantation to evaluate ridge development. Some of the host embryos were sacrificed and fixed in Bouin's fixative, paraffin embedded, serially sectioned

at 7 m, and stained with Milligan's Trichome stain. The remaining host embryos were allowed to develop for another ten days.

For the following experiments, right presumptive wings from stage 15-16 embryos were isolated and separated into dorsal and ventral halves. The anterior ends were marked with carbon to be certain of their orientation during transplantation. The tissues were grafted on the same stage host at a dorsal or ventral site on the right prospective leg or flank region according to the technique described in the preceeding paragraphs.

The third series of experiments involved isolation of dorsal and ventral mesoderm of the right presumptive wing from stage 14-16 donor embryos after incubation in 1% EDTA in calcium-magnesium free (CMF) PBS at 38°C for 20 minutes. Each half or two dorsal or two ventral halves were recombined with trypsin-isolated (1% trypsin in CMF-PBS for 1 1/2-2 hours at 4°C) whole limb-ectoderm from the same stage donors. The recombinants were grafted to the somite region of stage 19-21 host embryos.

All host embryos were returned to the incubator and allowed to develop for ten days, after which the hosts were sacrificed and the grafts recovered. Grafts were evaluated by their capacity to grow and become fully-developed wings.

Results

Development of Entire Limb Regions

When the presumptive limb mesoderms and their overlying ectoderm were transplanted to the host limb fields from which the ectoderm had been removed, two sets of results were observed depending on the orientation of the transplants relative to the hosts' axes (Table III-1). If the dorso-ventral axes of the donor and host coincide, the donor limb develops normally, regardless of the orientation of its antero-posterior axis (Fig. III-1). Fifty out of fifty-four transplants develop into relatively normal wings with axial polarities conforming to the orientation of the graft. When the limb fields were grafted with their dorso-ventral polarity reversed relative to the hosts', they fail to develop in forty out of sixty-three cases. The remaining twenty-three cases are poorly developed limbs. None formed into complete limbs with all three regions (stylopod, zeugopod, autopod) present. Whether the antero-posterior axis was normal or reversed made no difference in the development of these transplants.

Forty-eight hours after transplantation, some of the grafts were examined under the dissecting microscope for the presence or absence of the ectodermal ridge (Table III-2). Limb buds from grafts with their dorso-ventral axes reversed relative to the host's have either a rudimentary ectodermal ridge or no ridge at all. If the dorso-ventral axes of the graft and host correspond, the limb bud has a well-developed ectodermal ridge. The histology of the ectodermal ridge of experimental limbs confirms the earlier observation that the

overlap of two limb fields with opposed dorso-ventral polarities inhibited the development of the ectodermal ridge (Fig. III-2).

Development of Double Dorsal and Double Ventral Limbs

To test the necessity for normal dorso-ventral polarity in limb outgrowth, double dorsal and double ventral limb fields were created. Two dorsal or two ventral halves of presumptive limb mesoderm were placed side by side and bound together by a single limb ectoderm from the same stage donor. These composite limbs were transplanted to the somite region of stage 19-21 host embryos. Double dorsal recombinant limbs formed fully-developed wings in eleven out of seventeen cases (Table III-3). Similarly recombined and transplanted single dorsal halves developed in thirty-one out of forty-six cases.

Double ventral limbs transplanted to stage 19-21 hosts at the somite region developed in only four of seventeen cases while single ventral halves developed in three of thirty-one cases. It appears from these results that dorsal tissue has greater capacity to induce ridge and develop than ventral tissue. In all the above experiments, dorsal halves formed bidorsal wings and ventral halves into biventral limbs as diagnosed by the feather pattern.

Development of Dorsal and Ventral Halves of Presumptive Limbs

The above results are not consistent with the idea that dorso-ventral differences are necessary for limb development. They do suggest that the ventral half of the presumptive limb cannot develop autonomously on a non-limb site. To further evaluate the potential of

dorsal and ventral limb halves, their ability to develop after transplantation to the host limb field was examined. Presumptive wing regions were excised from donor embryos, cut into dorsal and ventral halves, and grafted to the leg region of host embryos on either dorsal or ventral position. Grafts of either dorsal or ventral halves to the dorsal region of the right prospective leg formed fully-developed wings (Table III-4). The dorso-ventral polarity of these wings are normal as evaluated by their feather pattern. On the other hand, grafts of the same pieces of tissue to the ventral region of the presumptive leg failed to develop. It appears that the dorsal limb region can promote outgrowth of the transplanted tissue while the ventral region may suppress the development of the graft (Figure III-3).

The ventral inhibitory influence appears to be specific for the limb field only. The dorsal halves of the presumptive wings transplanted to the dorsal or ventral flank region can develop autonomously. The ventral halves, however, developed into wings only when transplanted to the dorsal flank region but not on the ventral side. There are some cases where ventral halves formed outgrowths in the ventral flank region. However, these grafts appear to have overlapped the limb field of the host indicated by supernumerary digits attached to the host's limb.

Discussion

Two limb fields superimposed so that only their antero-posterior axes are opposed but their dorso-ventral axes are in accord form well-developed apical ectodermal ridges and subsequently fully-developed wings (apdd grafts). The wings formed have their antero-posterior and dorso-ventral axes conforming with the polarity of the donor. It has been suggested that the formation of the ridge and establishment of the proximo-distal axis require that both antero-posterior and dorso-ventral axes are determined in the presumptive limb (Meinhardt, 1982). The results suggest that elimination of dorso-ventral differences in the limb mesoderm by superimposition of two limb fields with opposing dorso-ventral axes interferes with the formation of the ectodermal ridge of the donor limb and inhibits limb outgrowth. The observed effects at stage 14-16 coincide with the period of ridge induction shown by earlier studies (Kieny, 1968; Saunders and Reuss, 1974; Carrington and Fallon, 1984). Other evidence, however, argues against the necessity for normal dorso-ventral differentials in limb outgrowth. Bidorsal limb fields develop, giving rise to bidorsal wings. Likewise, dorsal halves alone develop in the absence of ventral tissue.

Another possible explanation for the failure of dorso-ventrally opposed limb fields to develop is the existence of an inhibitor of limb outgrowth (possibly of ridge induction?) displaced dorsally or ventrally. The results of transplanting presumptive limb halves suggest that a ventral inhibitor exists. Dorsal halves which form

fully-developed limbs in dorsal limb or non-limb regions fail to develop when transplanted to a ventral limb site. This interpretation is complicated by the development of ventral halves grafted to a dorsal limb site. However, the autonomous development of early limb fields has been shown to depend on inductive influence from the adjacent somites (Pinot, 1970). The same somitic influence may be responsible for overcoming the ventral inhibition.

Our results also suggest the existence of a dorsal influence which promotes limb outgrowth possibly by inducing the formation of an ectodermal ridge. In the limb bud, the dorsal limb mesoderm may induce ridge formation with a responsive ectoderm experimentally (Carrington and Fallon, 1986) or in eudiplopodia mutants (Fraser and Abbott, 1971a, b). This morphogenetic influence may originate from the axial tissue such as somites (Kieny, 1970; Kieny *et al.*, 1972; Pinot, 1970; Agnash and Kochar, 1977) or neural crest (Shoobridge *et al.*, 1983). Barriers inserted between the axial tissues and the presumptive limb can cause deficiencies in the resulting limbs (Stephens and McNulty, 1981; Sweeny and Waterson, 1969). The morphogenetic influence from the axial tissues may account for the development of grafts transplanted to the dorsal region of either limb or flank. The influence, however, has been shown by Pinot (1970) to be weak in the flank region compared with the limb field. This may explain the well-developed wings obtained from grafts on the dorsal limb region as opposed to poorly developed wings from dorsal flank grafts. The ability of the dorsal half of presumptive wing to develop in the ventral flank region may

indicate that the dorsal tissue has already been exposed to the morphogenetic influence whereas the ventral half is not.

The relationship between dorsal and ventral influences and ridge induction and limb outgrowth described here and dorso-ventral polarity of muscles and skeletal elements in chapter I and II are not known. The ridge inducing ability of the dorsal mesoderm has been demonstrated under experimental conditions (Carrington and Fallon, 1986) or in eudiplopodia mutants (Fraser and Abbott, 1971a, b). Ventral mesoderm may show similar ridge-promoting characteristic when treated experimentally with a teratogen. This ventral ridge formation, however, seems to require damage to the overlying ectoderm (Scott et al., 1987). The results parallel the need for healing flank ectoderm for limb mesoderm to induce ridge and supernumerary outgrowth after transplantation to the flank of host embryos (Kieny, 1968; Saunders and Reuss, 1974). Ventral mesoderm can also maintain a ridge grafted to the ventral region of the limb bud (Saunders et al., 1976). The response of the ectoderm to ridge induction by the underlying mesoderm may depend to a certain extent on the character of the ectoderm. However, under normal conditions, a single ectodermal ridge is formed during limb development at the intersection of dorsal and ventral regions. The dorso-ventral differentials in the mesoderm may possibly determine the site at which the ridge will develop.

Our results show that differences exist in the dorsal and ventral regions of the presumptive limb in their capacity to promote outgrowth and limb development. Dorsal influences, which may originate from the somites, stimulate while ventral influences inhibit limb development.

It is probable that the site of limb initiation and possibly ridge induction is determined by the interaction of a dorsal (somitic) stimulation and a ventral inhibition, influences that may thus define the dorso-ventral boundaries of limb-forming regions.

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PART III

APPENDIX

Table III-1. Results of transplantation of presumptive wing to the prospective leg region of host embryos.

Orientation of the Graft Relative to the Dorso- Ventral Axis of the Host	Growth of the Limb		
	No Growth	Poor	Good
Dorso-ventrally Reversed	40	23	0
Normal Dorso-ventral Orientation	0	4	50

Table III-2. Ectodermal ridge development of composite limb buds 48 hours after transplanting a presumptive wing to the prospective leg region.

Orientation of the Graft Relative to the Dorso- Ventral Axis of the Host	Ectodermal Ridge Development		
	None	Small	Good
Dorso-ventrally Reversed	8	31	0
Dorso-ventrally Normal Orientation	0	3	18

Table III-3. Results of recombining dorsal or ventral presumptive limb mesoderm with whole limb ectoderm.

Mesoderm Donor	Donor Growth	
	Wing	No Wing
Dorsal Mesoderm	33	15
Ventral Mesoderm	3	28
Two Dorsal Mesoderms	11	6
Two Ventral Mesoderms	4	13

Table III-4. Results of grafting dorsal or ventral halves of presumptive wing to either dorsal or ventral region of the right prospective leg or flank.

Donor Tissue	Site on the Host	Donor Growth	
		Wing	No Wing
Dorsal Half Presumptive Wing	Dorsal Limb	24	0
	Ventral Limb	0	21
	Dorsal Flank	20	2
	Ventral Flank	12	5
Ventral Half Presumptive Wing	Dorsal Limb	28	0
	Ventral Limb	0	20
	Dorsal Flank	18	5
	Ventral Flank	5	13

Fig. III-1. Results of transplantation of presumptive wing on the right prospective leg region of the host in the following orientation relative to the host's axes (a) aadv (b) apdv (c) aadd (d) apdd. Grafted limbs (arrows) failed to develop when the dorso-ventral of graft and host are opposed (a, b). Note the well-developed wings formed when the dorso-ventral axes coincide (c, d).

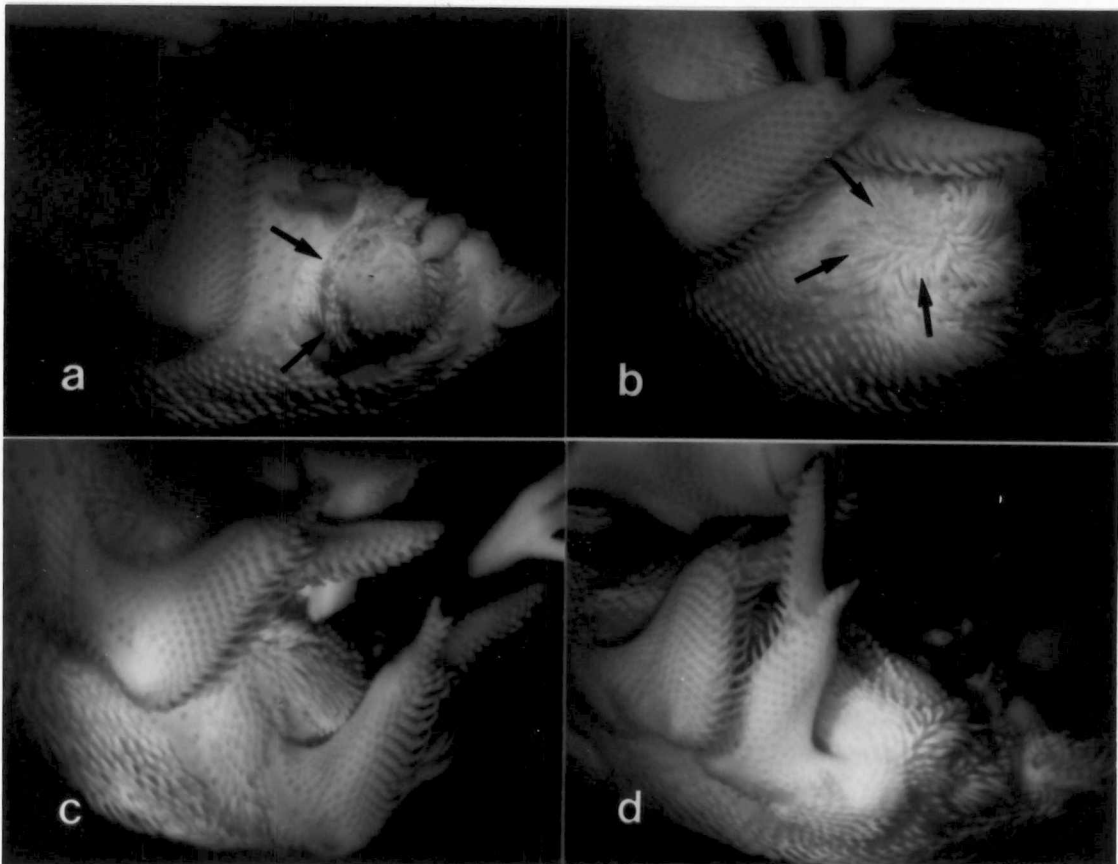


Fig. III-2. Histology of the ectodermal ridge 48 hours after transplantation of presumptive wing to the prospective leg region in the following orientation relative to the host's axes: (a) and (b) the dorso-ventral axes are opposed; (c) and (d) the dorso-ventral axes are in accord. Note the reduced or absent ridge (arrow) in (a) and (b) and the presence of well-developed ridge in (c) and (d) and in the contralateral limb-bud (arrow).

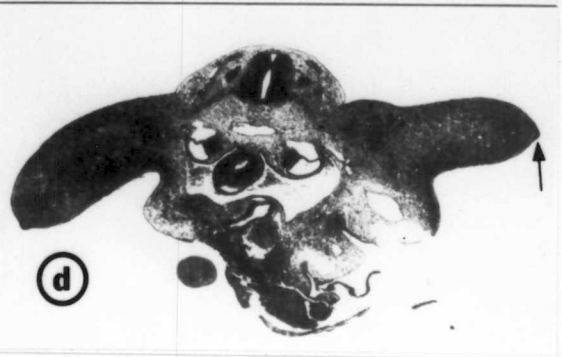
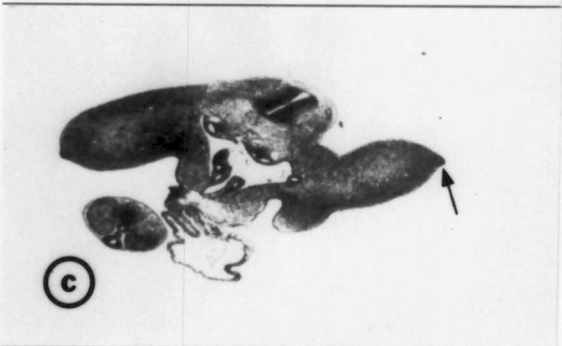
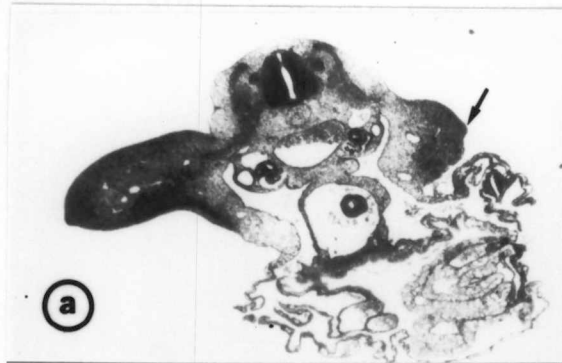
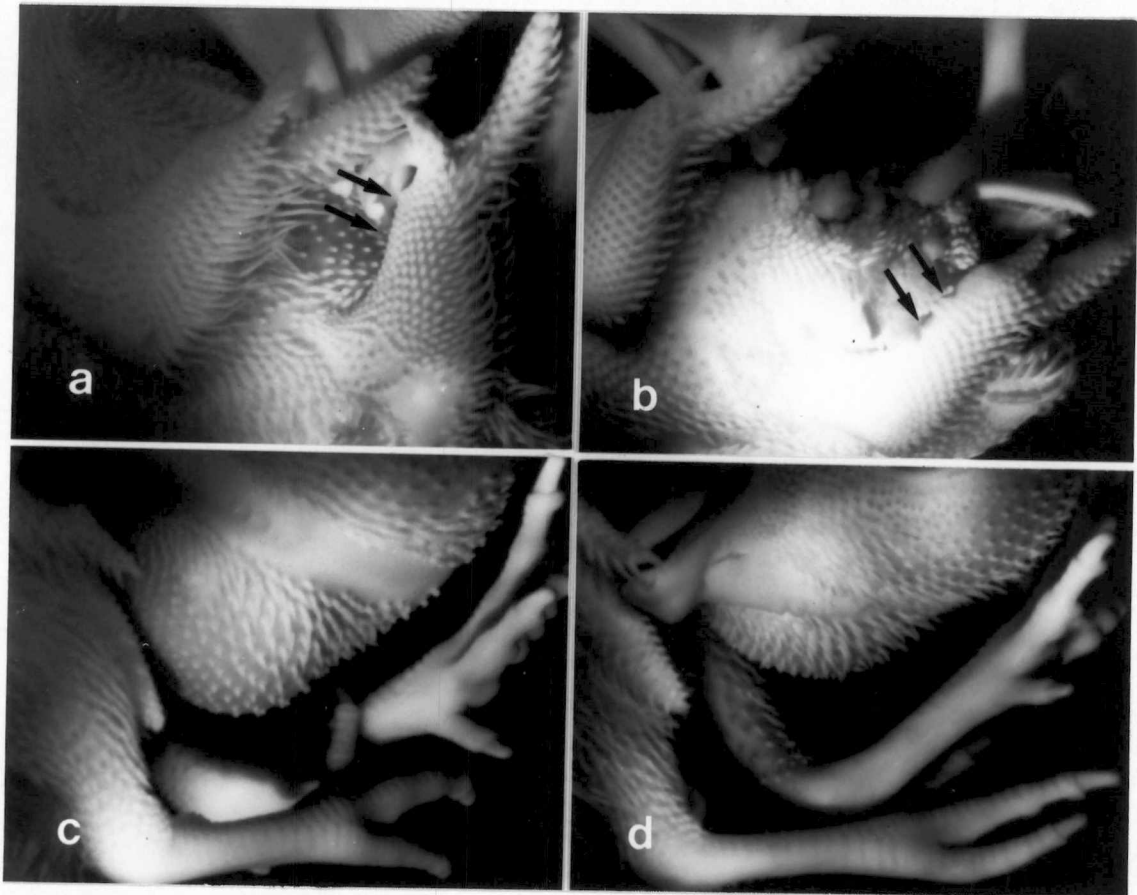


Fig. III-3. Results of grafting (a) dorsal and (b) ventral halves of presumptive wing to the dorsal region of the right prospective leg of the host. These formed fully-developed wings (arrows). Grafts in (c) and (d) are the same pieces of tissue transplanted to the ventral region of the limb field. On the ventral limb site, the grafts failed to develop but allowed formation of the host's leg.



GENERAL CONCLUSIONS

The spatial organization of structures along the dorso-ventral axis of the developing chick limb is under the control of both mesoderm and ectoderm. At the prelimb stages (stage 14 and younger), the control resides in the limb mesoderm. Dorso-ventral reorientation of the limb ectoderm relative to the mesoderm at these early stages always form wings with normal (mesodermal) dorso-ventral polarity. The results are in agreement with the earlier findings of mesodermal control of dorso-ventral polarity during these stages (Kieny, 1960; Kieny, 1968; Saunders and Reuss, 1974). At the onset of mesenchymal condensations at stage 15, designating the limb-forming regions, control of the dorso-ventral pattern shifts to the overlying ectoderm. At stage 15 and older, the limb ectoderm is polarized dorso-ventrally and can impose this polarity on the distal regions of the limb mesoderm. Therefore, dorso-ventral reversal of the limb ectoderm on a limb mesoderm starting at stage 15 results in bidorsal feather pattern and the reversal of muscle and skeletal elements in the autopod of recombinant wings. The ectodermal effect is observed at stages that previous studies have reported mesodermal control of dorso-ventral polarity (Kieny, 1968; Dhouailly and Kieny, 1972; Saunders and Reuss, 1974). The assessment of earlier investigators, however, were based on patterns of epidermal structures which in our results do not give a good indication of the ectodermal effect on mesodermal derivatives. In addition, the recombinations were done with flank ectoderm which has different properties than limb ectoderm.

The effect on mesodermal derivatives confirms what was earlier suggested in vivo to be the influence of the ectoderm, after its dorso-ventral reorientation, on cartilages based on the curvature of digits and flexion of the joints (Pautou and Kieny, 1973; MacCabe et al., 1973; MacCabe et al., 1974; Pautou, 1977). This is also consistent with the in vitro observations that the ectoderm can inhibit chondrogenesis of adjacent mesenchyme cells (Kosher et al., 1981; Solursh et al., 1981), a mechanism which possibly insures that cartilage formation would only occur at the core of the limb. We have carried the conclusion further by showing in vivo that the ectoderm does more than ensure the development of an endoskeleton; more importantly it controls the shape and spatial orientation of limb chondrogenesis. This ectodermal influence affects not only cartilages but also the pattern of muscles and tendons in the limb. The effect on soft tissues may be in the subdivision of the premuscle masses to form the individual muscles in the autopod. Dorso-ventral inversion of the ectoderm has modified this process, thus maintaining the asymmetry of muscle distribution according the new ectodermal orientation. This ectodermal effect on mesodermal derivatives is not surprising since the ectoderm has been shown in vitro to have an extensive zone of influence in the mesoderm (Solursh, 1984).

The normal dorso-ventral pattern of recombinant wings from stage 14 donors after dorso-ventral reorientation of the limb ectoderm indicates absence of dorso-ventral information in the ectoderm and/or failure of the mesoderm to respond to the ectodermal signal. We have evidence, in fact, that both these alternatives occur. Stage 14

ectoderms recombined in reversed dorso-ventral orientation with limb mesoderms which can respond to ectodermal influence (stage 15-17), develop into wings with mesodermal polarity. Recombinations of stage 14 mesoderm with older ectoderms (stage 15-17), which are capable of affecting mesodermal patterns of differentiation, also form wings with mesodermal polarity. The latter results suggest reprogramming of the ectoderm's dorso-ventral information by the mesoderm and imply that the source of information is the mesoderm. The double-recombination experiments confirm these conclusions. Ectoderm which resides in reversed dorso-ventral orientation on stage-14 mesoderm for 16 hours acquires reversed dorso-ventral information. It can thereafter cause muscle and cartilage reversals when combined with the mesoderm in normal orientation. This is effective with stage-14 but not with stage-16 mesoderm. It further shows that the dorso-ventral information transfer to the overlying ectoderm occurs for a brief period (about eight to ten hours) beginning at stage 14. The ectoderm's dorso-ventral information, however, is not stable until stage 18 and prior to this stage can be reprogrammed by a young mesoderm. The acquisition of dorso-ventral information by the ectoderm may possibly involve two stages. Initially the ectoderm obtains dorso-ventral information from the mesoderm, followed by a gradual stabilization of this ectodermal information.

The period of dorso-ventral information transfer to the ectoderm occurs at the same time that the ectodermal ridge is induced by the mesoderm (Kieny, 1968). The stabilization of the dorso-ventral information in the ectoderm at stage 18 also coincides with the

appearance of a recognizable change in morphology of the ectodermal ridge (Todt and Fallon, 1984). The timing of these interactions may be important and prompted the study on significance of dorso-ventral differentials in ridge induction and limb outgrowth.

Dorso-ventral polarity in the limb mesoderm was eliminated by grafting one limb field over another in reversed dorso-ventral orientation. This interfered with the formation of the ectodermal ridge and prevented the development of the limb. The effect is specific for the dorso-ventral axis since opposing the antero-posterior axes of two overlapping limb regions had no effect on ridge formation and the grafts formed well-developed wings. The dorso-ventral differences in the mesoderm, however, are not necessary for ridge induction. Dorsal mesoderm is capable of inducing ridge on limb ectoderm and forms a well-developed wing. This region can also promote good outgrowth of transplanted dorsal and ventral presumptive limb tissues. The ventral mesoderm, on the other hand, has limited capacity to induce ridge and inhibits outgrowth of grafted dorsal and ventral limb halves. The ventral inhibitory influence is specific for the limb field only. Thus in the presumptive limb region there is a dorsal influence which promotes outgrowth and a ventral inhibition of outgrowth. The possible source of this information is the somites (Kieny, 1969; Pinot, 1970). The dorso-ventral differences in the limb although not necessary for ridge induction may be involved in determining the site at which the ridge will develop.

Several models have been proposed to account for the mechanism of pattern formation in the limb (Wolpert, 1971; Tickle *et al.*, 1975;

Wolpert et al., 1975; French et al., 1976; Newman and Frisch, 1979; Bryant et al., 1981). Among these, two models are widely accepted to account for patterning in the chick limb. The models share the concept that cells have positional information to determine their pathway of cytodifferentiation, but differ in how positional information is generated.

The ZPA/progress zone model by Wolpert et al., (1975), proposed that cells obtain positional values along two coordinates, the antero-posterior and proximo-distal axes. The information along the proximo-distal axis is specified by the length of time cells spend in the progress zone under the influence of the ectodermal ridge. Cells leaving the progress zone early are assigned more proximal values. Position along the antero-posterior axis is specified by the zone of polarizing activity (ZPA), the source of a diffusible morphogen distributed in a gradient along this axis with its highest concentration at the posterior region. Although the model does not account for pattern formation along the dorso-ventral axis, Wolpert (1981) has suggested that each of the axes in the limb may be specified by a different mechanism. Complimenting this concept, we have presented evidence that the dorso-ventral axis is controlled by both mesoderm and ectoderm. At early stages of development control resides in the mesoderm with a subsequent shift of influence to the overlying ectoderm as limb development progresses. The ectodermal influence, however, is confined only to the distal region of the limb where cells can change their positional values.

The polar coordinate model, developed from regeneration experiments in vertebrates and invertebrates (French et al., 1976; Bryant et al., 1981), has been applied to studies on limb development in chickens (Iten, 1982; Javois, 1984). According to this model, cells have positional information specified in two coordinates: a circumferential set of values specifying the transverse axis (including both antero-posterior and dorso-ventral axes) and radial values determining position along the proximo-distal axis. The coming together of cells with different positional values could lead to growth and restoration of the missing values by intercalation. This model attempts to explain patterning along all axes by a single common mechanism, whereby positional information for pattern formation resides in the mesoderm and the ectoderm exerts no control (Bryant and Iten, 1976; Bryant et al., 1981; Javois, 1984). The present results has demonstrated quite significant effects of the ectoderm on dorso-ventral pattern. It is possible that in the chick limb a totally different mechanism for pattern formation exists. Such differences may account for the capacity of amphibian limbs to regenerate and failure of chick limbs to do so. The model can account for the present results if the assumption is changed and positional information is attributed to the ectoderm. The 180° rotation of the ectoderm would, therefore, change the dorso-ventral positional value of the underlying mesoderm and result in reversal of muscle and cartilage patterns.

The study has demonstrated that the control of pattern formation along the dorso-ventral axis involves both limb mesoderm and ectoderm. It has clearly demonstrated quite significant effect of the ectoderm

on mesodermal dorso-ventral patterns. Initially the information is in the mesoderm but at the onset of limb development dorso-ventral information is transferred to the ectoderm. Although similar interactions may occur between ectoderm and mesoderm in the establishment of other axes in the limb, the dorso-ventral patterning is unique because of this information transfer and the shift from mesodermal to ectodermal control. Proximo-distal and antero-posterior patterns, on the other hand, are controlled by the mesoderm throughout limb development. Finally, the results of transplanting whole and half limb fields suggest the existence of a ventral inhibition of ridge induction and limb outgrowth. They also show a dorsal stimulation of limb development which probably coincides with a previously demonstrated somitic inducer of limb outgrowth. The interaction of the dorsal inducer and ventral inhibitor may be involved in determining the dorso-ventral boundaries of the limb field.

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