Ultrastructural features of ectodermal–mesenchymal relationships in the developing limb of *Xenopus laevis*

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**SUMMARY**

Electron microscopical study of the formation of the hind limb of the toad *Xenopus laevis* between stages 49 and 53 of larval development has been performed with special reference to the relationships between the apical ectodermal ridge and the underlying mesenchyme. It has been found that the intervening collagenous layer is thinner in the vicinity of the ridge than elsewhere and that the subjacent mesenchymal cells are more loosely arranged. There are also fewer mesenchymal filopodia crossing the collagenous layer than proximally although the distribution of delicate vertically disposed threads, also observed at the ecto-mesenchymal junction, is uniform over the entire bud. Overall, it is concluded that the fine structural features of *Xenopus* limb development, during these stages, do not give clear insight into the mechanisms by which the apical region exerts its morphogenetic effects.

**INTRODUCTION**

It has recently been shown that the limb-buds of *Xenopus laevis* possess an apical ectodermal ridge (Tarin & Sturdee, 1971), although in Amphibia this structure is less prominent than that found in many other vertebrates.

The ridge is thought to play an inductive role in limb morphogenesis since the results of numerous experiments performed on the chick limb suggest that it promotes the outgrowth of the limb mesenchyme and controls the orientation of the paddle (see Zwilling, 1961, for review). Comparable experiments on limb development in *Xenopus* indicate that the ridge exercises a similar morphogenetic function in Amphibia (Tschumi, 1957). However, this view is contested by others (see Amprino, 1965, for review) who deny that the ridge is inductive and consider that its effects on the limb mesenchyme are merely mechanical or structural in nature. In the latter scheme it is envisaged that the ridge forms a stiffened border to the limb-bud, thereby passively moulding its shape, while the morphogenetic functions attributed to the ridge in fact reside in the apical mesenchyme.

In connexion with the former interpretation (i.e. that the ridge plays an

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inductive role) the results of ultrastructural studies performed on limb-buds of the mouse and chick by Jurand (1965) and of the chick by Bérczy (1966) require consideration. These authors noted that the cells of the ridge, and the ecto-mesenchymal junction beneath them, displayed special features not found elsewhere in the bud, although Jurand prudently observes that such findings alone can neither prove nor disprove an inductive role for the ridge.

Electron microscopical studies of other systems where inductive interaction is known to occur have revealed several properties which may be characteristic of such phenomena (Grobstein & Cohen, 1965; Kischer, 1968; Slavkin et al. 1969; Saxen, 1971). These features include:

(i) Regular and compact arrangement of cells in the epithelium and the mesenchyme.
(ii) Close proximity between the interacting tissues.
(iii) Small quantities of collagen at the interface between them.
(iv) Numerous filopodia and delicate amorphous threads approaching and contacting the lamina densa from the mesenchymal aspect.

The present ultrastructural survey was undertaken to discover whether similar morphological features are present during limb development in Amphibia, and to identify any additional features which might indicate the mechanism of interaction between ectoderm and mesenchyme, particularly at the apex of the limb-bud. We have therefore examined the hind limb-buds of larvae of *Xenopus laevis* from stages 49 to 53, during which phase the ridge first appears, reaches its maximum size, and then regresses (Tarin & Sturdee, 1971).

**MATERIALS AND METHODS**

Tadpoles of *Xenopus laevis* were reared and staged according to the methods described by Nieuwkoop & Faber (1967).

Three larvae were selected for study from each of stages 49 and 50, 5 from stage 51, and 2 each of stages 52 and 53, making a total of 15. These were anaesthetized using MS222 (Sandoz Products Ltd., London) and the trunk segments containing both hind limb-buds were cut out under a dissecting microscope. These smaller pieces of tissue were fixed at 4 °C for 2 h in 4 % glutaraldehyde buffered at pH 7·35 with 0·2 M sodium cacodylate.

The specimens were then washed in three changes of a mixture of equal volumes of 0·2 M sodium cacodylate and 0·44 M sucrose for 24 h at 4 °C. During this period the limb-buds were carefully dissected from the trunk and one of each pair was orientated in molten agar (45 °C) using a method similar to that described by Scott, Sharp & Tarin (1970). The small limb-buds of stages 49 and 50 were not separated, and thus the trunk segments with both limbs were embedded in agar. The specimens were orientated to provide ventral longitudinal sections of the bud (i.e. transverse sections of the ridge).

The limb-buds were post-fixed in cold 2 % osmium tetroxide for 2 h,
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Dehydrated in a graded series of ethanols, and embedded in Araldite. Thick sections cut on an LKB Ultramicrotome III were stained with 1% toluidine blue in 1% borax, and examined with an optical microscope (Fig. 1). Thin sections were taken from the midline of the bud (thus cutting the ridge, when present, at its highest point) and collected on single hole grids by the method of Galey & Nilsson (1966). These were double stained with 25% uranyl acetate in methanol and 0.4% lead citrate in 0.1 n-NaOH, and examined in an AEI EM 6B electron microscope.

RESULTS

The ectoderm of the limb-bud

At stage 49 the ectoderm contained a mixed population of light and dark cells distributed in two layers, a basal or sensorial layer and an outer or peridermal one. The cells of the former were spherical or cuboidal, while those of the outer layer tended to be flattened (Fig. 3). During subsequent stages the ectoderm became three-layered at the apex, and this, together with the assumption of columnar shape by the cells in the basal layer, contributed to the formation of the apical ectodermal ridge (Figs. 1, 4). A common feature of the ridge in histological sections, although not exclusive to it, was the presence of vacuoles and dense particles (Fig. 1). When examined with the electron microscope it was found that such appearances represent phagocytosed inclusions consisting of cellular debris (Fig. 2) which is presumably all that remains of effete cells ingested by healthy ectodermal cells (see also Kelley (1973) for similar appearances in human limb development).

The apical ectodermal ridge was thus clearly distinguishable from the surrounding ectoderm on the basis of its gross characteristics. However, the ultrastructure of the cells comprising the ridge did not differ significantly from that of those elsewhere on the limb-bud. There was for instance no evidence of special accumulations of cytoplasmic organelles or granules in cells of the ridge as a whole nor within parts of individual cells comprising this structure. Furthermore there were no obvious differences in the type or frequency of intercellular contacts (see Fig. 12), between cells in the ridge and those proximal to it, although in the more distal parts of the bud cells tended to lie closer together than elsewhere.

In general, the fine structure of ectodermal cells of the limb was similar in all five stages examined, although there were consistent differences between cells of the two layers. Those in the basal layer appeared to contain more rough endoplasmic reticulum than those in the outer one (e.g. Fig. 3), but in both instances it was uniformly distributed throughout the cytoplasm. In cells of the peridermal layer, mitochondria and Golgi bodies were located mainly on the external aspect of the nucleus close to the mucous vesicles (Fig. 2) of the free ectodermal surface (Figs. 3, 5). Tonofilaments were sparsely distributed in the peripheral regions of most cells and were often attached to desmosomes. These
latter structures were common, although other types of intercellular junction (such as described by Farquhar & Palade (1965) and Hay (1968) in other tissues) were also observed (see Fig. 12). The plasma membranes of adjacent ectodermal cells were not tightly interdigitated and intercellular spaces were a prominent feature of this epithelium (Figs. 2–5).

The ectoderm of the flank

Close to the base of the bud the ectoderm of the flank contained a remarkable number of tonofilaments located mainly in the cells of the sensorial layer (Fig. 10). Many bundles of these filaments were attached to the dense plaques of the hemidesmosomes. The latter were abundant along the inner plasma membrane of the basal cells and fine filaments extended from the hemidesmosome across to the lamina densa. In contrast, the ectoderm of the bud rarely displayed hemidesmosomes (Figs. 6, 7), although occasionally slight thickenings of the basal plasma membrane were noted, and possibly represented an early stage in the development of such structures.

Large pale cells (Leydig cells) were commonly observed in the flank ectoderm (and, from stage 51 onwards, in the proximal portion of the bud). The ultrastructural features of these have been described by Kelly (1966a).

In its other features the ectoderm of the flank resembled that of the bud.

The mesenchyme

There was little variation in the fine structure of the mesenchyme during the stages examined, except in areas such as those forming cartilage at stages 52 and 53.

A typical mesenchymal cell in section consisted of a large nucleus surrounded by a relatively small area of cytoplasm (Fig. 5). Within the latter, mitochondria,
Golgi bodies and profiles of endoplasmic reticulum were evenly distributed in a dense granular ground substance. Occasionally stacks of microfilaments (or tubules) and membrane-bound inclusions resembling lysosomes were observed.

Despite this ultrastructural uniformity there was great variation in the shape of the cells and in their proximity to one another. At stage 49 (Fig. 3) the cells were tightly packed and lay very close to the distal ectoderm. The plasma membranes of adjacent mesenchymal cells were tightly apposed with often less than 10 nm between them. In contrast, cells distant from the ectoderm, such as those in the proximo-central region of the bud, were widely scattered. The intercellular spaces contained numerous filopodia, often several micrometres long, which frequently made close contact with those of neighbouring cells. The intercellular regions also contained myelin figures and patches of unidentified cellular debris. In addition, groups of collagen-like fibres were commonly found both in areas near the ectoderm and amongst the cells in deeper regions of the mesenchyme.

At later stages cells in the distal and apical mesenchyme became less crowded (Figs. 4, 5), although those beneath parts of the lateral and medial ectoderm, further proximally, remained tightly grouped. Other sites where condensation of cells were noted include those in association with nerve fibres and in the central precartilaginous zones. Although the mesenchymal cells were well dispersed subjacent to the apical ectodermal ridge, by stage 51 they tended to be grouped about the marginal sinus. Cytoplasmic processes were numerous, but seldom as long as those observed near the base of the bud.

Degenerating or necrotic cells were not found in the mesenchyme, although cells whose cytoplasmic and nuclear appearances were atypical (Fig. 11) were occasionally observed.

There were no mesenchymal cells found in association with the ectoderm of the flank.

**FIGURES 4, 5**

Fig. 4. Apex of the hind limb-bud at stage 51. The cells in both the ectoderm (E) and mesenchyme (M) are much smaller than at stage 49 (Fig. 3) and the collagenous layer (CL) is wider. In the basal layer of the ectoderm the columnar cells of the ridge are clearly visible. The mesenchymal cells are widely dispersed and possess numerous fine cytoplasmic extensions (arrows). × 6000.

Fig. 5. Apex of the hind limb-bud at stage 53. The collagenous layer is still wider and its constituent fibres are more regularly arranged than previously. The mesenchymal cells remain well separated and the ectoderm has reverted to a two-layered structure following the regression of the ridge. × 6000.
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Ectodermal–mesenchymal junction

(a) Of the bud

The region dividing the ectoderm from the mesenchyme comprised the lamina lucida, the lamina densa, and the collagenous layer (Figs. 6, 7).

The lamina lucida was an electron-translucent space, 30–50 nm wide, which separated the ectodermal cell membrane from the electron-dense band of the lamina densa. This latter, 15–20 nm wide, formed the boundary of the collagenous layer which occupied most of the space between ectodermal and mesenchymal cells.

The total width of this junctional zone varied from 0.5 to 5 µm. It tended to be narrow adjacent to regions of closely packed mesenchymal cells, while at every stage it was wider along the lateral and medial sides of the bud than distally. The junction was narrowest at stage 49 at the distal extremity of the bud where the mesenchyme was densely packed beneath the ectoderm (Fig. 3).

The lamina densa was continuous on the medial, lateral and apical borders of the bud at all stages studied. Minute examination of this structure below the apical ectodermal ridge failed to reveal any discontinuities.

At stage 49 there were few sub-epithelial collagen fibres and these were not regularly aligned. Their scarcity and lack of orientation were most obvious in the distal zone of the limb. This disparity between the proximal and distal regions was maintained in subsequent stages, even though overall the quantity of fibres increased and their arrangement became more orderly.

The collagenous layer was traversed by slender threads, singly and in bundles, orientated at right angles to the lamina densa (Figs. 6, 7, 9). They were rarely seen at stage 49 but were common by stage 53. They did not appear to be either more, or less, frequent beneath the apical ridge. Many of these threads or filaments apparently crossed the lamina densa, and also the lamina lucida, and thus made contact with the ectodermal cell membrane. Others merged with the lamina densa, and the site of contact was marked by increased electron density (Fig. 9). Such attached threads frequently gave the appearance of being coiled round one another like the strands of a rope. Their origin and mode of formation is unknown.

Figures 6, 7

Fig. 6. The collagenous layer (CL) of the bud, midway between the apex and the base at stage 51. In contrast to the apex (compare with Figs. 3–5) the collagenous layer contains several mesenchymal filopodia (large arrows). Numerous threads (small arrows) orientated at right angles to the collagen fibres, approach and contact the lamina densa (Ld). × 18000.

Fig. 7. A comparable area to that shown in Fig. 6, but beneath the apical ectodermal ridge at stage 51. The collagenous layer is not as wide and does not contain any mesenchymal filopodia. Threads (small arrows) are in contact with the lamina densa (Ld). × 18000.
Another prominent feature of the collagenous layer was the presence of the filopodia extending from the mesenchymal cells (Figs. 6, 8). Many of these cytoplasmic processes crossed the entire width of the collagenous layer and contacted the lamina densa, although none were seen to extend into the lamina lucida. They frequently contained fine filaments orientated parallel to the long axis (Fig. 8).

Few filopodia were observed in the collagenous layer at stage 49. Subsequently they became common along the lateral and medial sides of the bud, but remained rare in the apical zone (Fig. 7).

(b) Of the flank

The collagen fibres of this region were arranged in a typical orthogonal pattern comprising numerous layers (Fig. 10). Tangential sections revealed that some fibres were apparently inserted into the lamina densa.

The collagenous layer of the flank was not traversed by either filopodia or the threads described above. This is perhaps not surprising since there were no mesenchymal cells in the vicinity.

A feature worth note was the presence of small electron-dense bodies disposed in a single row like a string of beads (Fig. 10) in the lamina lucida. The shape of the beads varied from circular to ovoid and some appeared rectangular. The longest axis ranged from 15 to 40 nm and was generally orientated so that the beads did not touch either the lamina densa or the ectodermal cell membrane. Examination at higher magnification revealed (Fig. 13) that many beads possessed striations lying approximately parallel to the lamina densa. The width of the dark bands was within the range 1·0–2·0 nm while the pale bands were slightly narrower.

Studies of the distribution of the beads showed that they extended as far as the base of the bud and ceased abruptly as soon as the limb ectoderm began (compare Figs. 6, 7 and 10).

Their function and origin are unknown.
Fig. 11. Low-power view of two atypical mesenchymal cells found close to the ectoderm of the proximo-lateral part of the bud. Note the separation (small arrow) of the two layers of the nuclear envelope, and the condensed chromatin within. × 8000.

Fig. 12. Examples of intercellular junctions observed in the limb ectoderm.
(a) Desmosome (macula adhaerens) (×40000) from the proximal portion of the limb-bud.
(b) Modified desmosome (×40000) from the apical ectodermal ridge. The thickened plaques on the cell membranes and the moderately dense intercellular substance are easily seen but there are no tonofilaments or linear densities between the apposed cell membranes (see Fig. 12a).
(c) Gap junction (×40000) from the apical ectodermal ridge. The apposed membranes are thicker and more clearly delineated than cell membranes elsewhere.
(d) Tight junction (fascia occludens) (×40000) from the flank. These are found in relation to the external surface of the skin; between the outer portions of superficial ectodermal cells.

All the above types of intercellular junctions were present both in the ridge and in the proximal ectoderm and their structure was identical in the two regions.
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DISCUSSION

This work shows somewhat surprisingly that there are no obvious ultrastructural features in the apical ectoderm or mesenchyme which distinguish them from similar tissues elsewhere in the bud. We could find no record of any other electron microscopical study of amphibian limb development and were therefore limited to comparing our observations with those obtained in similar studies on chick (Jurand, 1965; Bérczy, 1966), mouse (Jurand, 1965) and human (Kelley, 1973) limb formation. Two of these (Jurand, 1965; Bérczy, 1966) report that the apical ectodermal cells have special cytodifferentiative features which distinguish them from the ectoderm elsewhere. The cells in the ridge were found to contain more endoplasmic reticulum, mitochondria and Golgi bodies. Also Bérczy reported that zonula occludens (‘tight’) intercellular junctions were present only in the ridge and that the number of pinocytotic vesicles on the basal plasma membrane was greater here than elsewhere.

Our interest has focused mainly on the interfacial region between the two tissues, which is considered more fully later, but so far as the internal structure of the cells is concerned we found that the junctions between the cells and the organelles within them were qualitatively much the same as anywhere else in the bud. Detailed quantitative studies were not undertaken but there were no obvious differences in the proportions of various organelles in the two tissues in different regions.

In the apical mesenchyme we did not find any distinctive ultrastructural features. Nor did we find any evidence amongst the cells nearest the ectoderm of polarization of intracellular organelles towards the ectodermal aspect of the cell. The close packing of the cells beneath the ectoderm in the early stages of limb formation is similar to the packing of mesenchymal cells reported in other
organs during epithelial–mesenchymal interactions (Saxen & Wartiovaara, 1966; Koch, 1967, fig. 18; Tarin, 1971) but this phenomenon is not confined to the apical region of the bud. Also the close packed cells are dispersed by stage 51 when the epidermal ridge attains its maximal development. However, it is important to note that similar accumulations of mesenchymal cells do not occur under the flank epidermis and such features might therefore still be linked with inductive interrelationships in limb development.

The marginal sinus, which always follows the same course as the ridge, was devoid of a surrounding lamina densa during these stages and lay some 20–30 \( \mu \text{m} \) deep to the ectodermal thickening. Thus, any interrelationships between these two structures, involved in achieving correspondence in their courses, must be independent of cellular contact and capable of operating across a considerable distance. It is worth mention in this respect that there were no special morphological features in the intervening space.

The significance of the atypical cells occasionally observed in the mesenchyme is unknown but the condensation of nuclear chromatin indicates that either they are preparing for mitosis or that they are in the early stages of degeneration. These cells are similar to those described by Kelley (1970) and by Hammer & Mottet (1971). Like the former we favour the interpretation that the cells are preparing for mitosis (perhaps in early prophase) because, in our preparations, cytoplasmic features such as mitochondria were apparently in good condition.

The interface between the ectoderm and the mesenchyme was the subject of special interest in this work because studies on other developing organ systems such as the pancreas (Kallman & Grobstein, 1964), the salivary glands (Grobstein & Cohen, 1965) and the tooth (Slavkin et al., 1969) have shown the presence of ultrastructural features which are possibly implicated in developmental interactions between the tissues involved. These features include fine collagen fibrils near the epithelial surface, mesenchymal filopodia contacting the ectoderm and vertically disposed fine filaments or threads and may be present individually or in combination depending on the organ involved. In the amphibian limb all of these features were present at the interface but were not confined to the vicinity of the apical ectodermal ridge. In fact, quite to the contrary, in this part of the bud mesenchymal filopodia rarely approached the ectoderm and the subepidermal collagen layer was much thinner, although vertical threads were abundant in all regions. It is therefore appropriate to consider the possible significance of each of these interfacial features individually.

So far as collagen fibrils are concerned it has been shown that during salivary gland development these accumulate around the stalks of the epithelial rudiments and are absent from the tips of the growing adenomeres (Grobstein & Cohen, 1965). Moreover, treatment of explanted rudiments with collagenase was found to cause retarded and abnormal development but this procedure did not usually cause complete cessation of morphogenesis (Grobstein & Cohen, 1965). Disappearance of the subepidermal collagenous lamella was also noted.
by Balinsky (1957) in supernumerary limb induction by transplanted otic placodes in amphibian embryos. Similar observations were recorded in limb regeneration in adult amphibians by Thornton (1954), who found that the apical cap forms only in an area where dermal fibres have retracted and resorbed. Salpeter & Singer (1960) reported that regeneration will not ensue if the epidermis is separated from the connective tissue cells by a barrier of connective tissue fibres.

It seems likely therefore that collagen fibrils play no direct initiatory role in inductive interactions (see also Wessells & Cohen, 1968), but in the limb and elsewhere their distribution may, by exerting a mechanical effect, be responsible for the shaping of organ rudiments. It has also been suggested by Grobstein & Cohen (1965) that collagen fibres may play a role in developmental interactions by screening off some areas to prevent further epithelial–mesenchymal communication but this hypothesis probably does not apply unmodified to the limb of *Xenopus*, since collagen is present at the epithelial–mesenchymal junction over the entire surface of the bud, although there is less at the apex than elsewhere. It is pertinent in this context to recall Kelley’s (1973) observation that in the developing human limb collagen-like fibrils were prevalent close to the epithelium in the interdigital zones of necrosis but not at the tips of the digits where the epithelium was stratified and thickened.

The chemical composition and significance of the vertically disposed threads observed in some inductive systems (Kallman, Evans & Wessells, 1967; Kischer, 1968; Slavkin *et al.* 1969) are still obscure and there is no direct evidence that they are involved in developmental interactions. In the amphibian limb their presence both at the apex and elsewhere indicates that they are not concerned with inductive effects of the apex on the development of the bud (see Introduction). However, it is possible that they may have no significance in communications between the two tissues and may exercise an entirely mechanical function such as tethering the ectoderm to the deeper tissues.

The significance of the filopodia is also unknown and, once again, in spite of their presence at the epithelio–mesenchymal junction in other organ rudiments, there is no direct evidence that they are involved in inductive interactions. However, their rarity in the vicinity of the ridge provides an important though somewhat negative distinction between this region and the rest of the bud. Perhaps as with collagen this is an indication that they have a function in controlling or diminishing ectodermal morphogenetic activity.

The lamina densa and basal plasma membrane of the epidermal cells possessed no special features in any part of the bud. Thus, in *Xenopus*, we could not confirm Jurand’s (1965) observations of discontinuities in this structure beneath the apical ectoderm of the (chick) bud. It is relevant to mention that Bérczy (1966) and Kelley (1973) also failed to find such features in the chick and human respectively, and for the time being it is perhaps prudent to assume that they were the result of preservation artifacts.
The absence of 'beads' in the space between the ectodermal membrane and the lamina densa was a feature characteristic of the bud as a whole for which there is at present no satisfactory explanation. In embryonic amphibians they are also absent in the gills and balancers (Kelly, 1966b; Anderson & Kollros, 1962).

It is appropriate to mention that these structures have been reported in the skin of several larval amphibians (see Kelly, 1966b, for review) and fish (Nadol, Gibbons & Porter, 1969) and that in these animals they disappear during metamorphosis.

To conclude, it is apparent that in the limb-bud of *Xenopus* there are no obviously significant fine structural differences between the apical region and more proximal zones. This indicates that the special morphogenetic activity attributed to the apical ridge-mesenchyme complex does not depend on morphological features detectable with the electron microscope. It should be noted however that this evidence is also compatible with the interpretation that in amphibians this complex exerts no more controlling influence over limb development than other regions of the bud.

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