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Myogenic cell movement in the developing avian limb bud in presence and absence of the apical ectodermal ridge (AER)

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SUMMARY

Fragments of quail wing bud containing myogenic cells of somitic origin and fragments of quail sphlanchopleural tissue were introduced into the interior of the wing bud of fowl embryo hosts. No movement of graft into host tissue occurred in the control, but myogenic cells from the quail wing bud fragments underwent long migrations in an apical direction to become incorporated in the developing musculature of the host. When the apical ectodermal ridge (AER), together with some subridge mesenchyme, was removed at the time of grafting, no such cell migration occurred. The capacity of grafted myogenic cells to migrate in the presence of AER persists to H.H. stage 25, when myogenesis has begun, but premyogenic cells in the somites, which normally migrate out into the early limb bud, do not migrate when somite fragments are grafted into the wing bud. Coelomic grafts of apical and proximal wing fragments showed that apical sections of quail wing buds become invaded by myogenic cells of the host, but grafts from proximal wing bud regions do not.

INTRODUCTION

Contrary to the impression given by histological sections, the developing limb bud represents a community of cells in a state of high activity. In 1969, Ede & Law pointed out that in order to obtain a reasonable computer model of limb development it was necessary to introduce a cell movement component, and in 1974, Ede, Bellairs & Bancroft described the apical mesenchyme cells in a SEM study as probably hauling themselves actively towards the limb apex by means of filopodia which attached to the basal lamina of the apical ectoderm. In the formation of the skeletal rudiments Holmes & Trelstad (1980) and Ede & Wilby (1981) have shown that the prechondrogenic cells orientate themselves towards, and probably move towards, the long axes of the precartilage condensations. Cells which do not originate in the somatopleural swelling also enter the limb

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bud, including the spinal nerve axons, led by their growth cones (Lewis, 1978), neural crest cells which differentiate as melanocytes and Schwann cells, and premyogenic cells which migrate out from neighbouring somites (Christ, Jacob & Jacob, 1974; Chevallier, Kieny & Mauger, 1977) to form the limb muscles. Cell movement, therefore, plays a widespread and considerable role in limb morphogenesis, and it is important to discover as much as possible about the factors which control and affect it.

The principle of most of the experiments reported here was to graft, *in vivo*, into the wing bud of the fowl, a fragment of quail wing bud and observe the subsequent distribution of quail cells within the fowl limb at a later stage. Almost all of the cells which were found to have moved were the myogenic cells of somite origin, and this study reports our observations on some aspects of the control of this myogenic cell movement within the developing wing.

MATERIALS AND METHODS

The experiments use embryos of the fowl (*Gallus gallus*, White Leghorn) and the Japanese quail (*Coturnix coturnix japonica*) both obtained from the Institut d'Embryologie's suppliers at Strasbourg. The stages at the time of operation are indicated according to the normal table of Hamburger & Hamilton (1951).

Experimental series 1

The principle of these experiments is to introduce into the interior of the fowl limb bud a fragment of quail limb bud which contains myogenic cells of somitic origin, either in process of migration or capable of migrating. For this purpose a fragment of the host limb was removed by means of an 'emporte-pièce', whose design has been described by Hampé (1959), which incorporates a circular knife 0·4–0·5 mm in diameter. The fragment was either cut out in the emporte-pièce or dissected out with steel needles. It was then transferred to a Petri dish containing Ringer's solution and trimmed to make its dimensions compatible with the hole made in the host wing bud. The experimental procedure is shown in Fig. 1A. Donor embryos (quail) were of stage 18–25 (H.H.); host embryos (fowl), stage 22–25 (H.H.). The graft was not orientated in any particular way.

As controls, the tissue fragment was taken from the splanchnopleure of $2\frac{1}{2}$-day quail embryos, or from the digestive tract (splanchnopleure or splanchnopleure less endoderm obtained by disassociation with 0·3 % Worthington CLSPA collagenese in Tyrode) of a 5-day embryo. Fixation was 3–4 days from the time of incubation. These tissues are comparable in being largely composed of mesenchymal, including muscle, cells, but none of these cells have been shown to migrate at any stage. If the limb myogenic cells in our experiments were moved passively, then these control cells should also be displaced in the experimental situation.

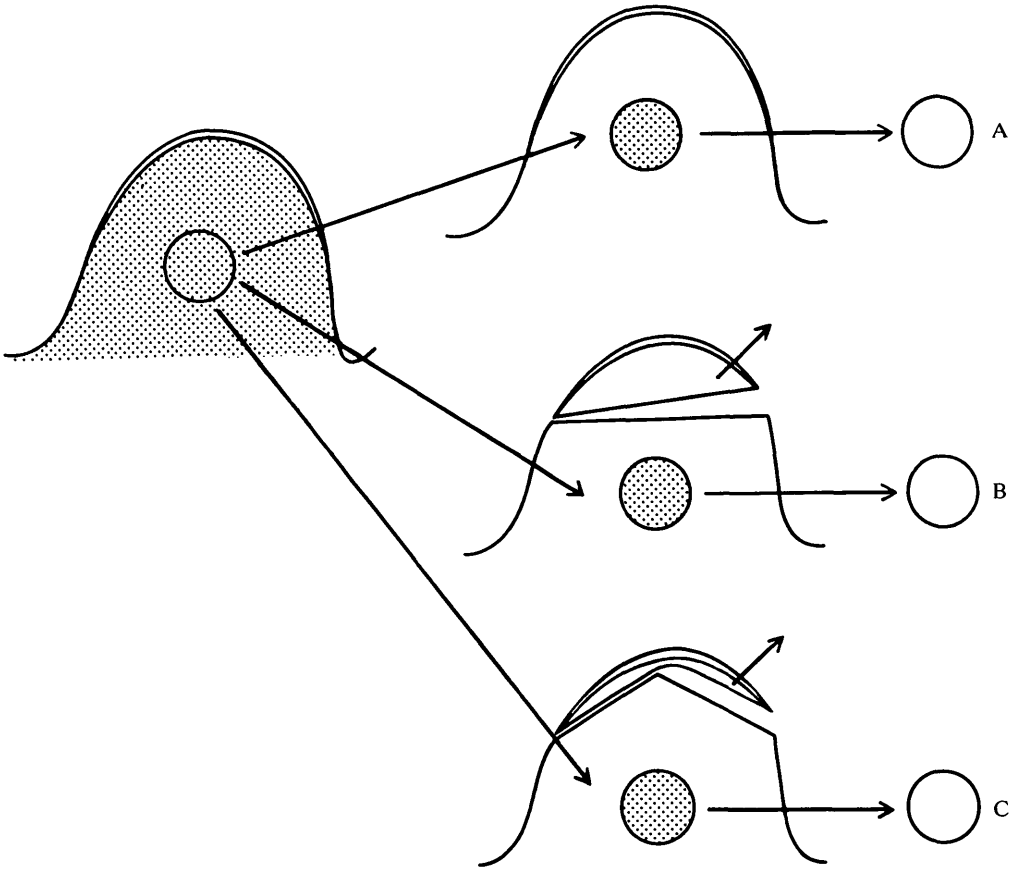

Fig. 1. Method of grafting fragment from quail limb bud (hatched) into chick limb bud *in situ*. Further explanation in text.

Host limbs were harvested after 2–4 days further incubation, fixed in Carnoy's fluid, serially sectioned in wax and stained by the method of Feulgen & Rossenbeck (1924) which makes it possible to distinguish quail from fowl cells (Le Douarin, 1973). In analysing the results of experiments each section was examined and the extent of any cell displacement away from the graft scored for the whole limb according to the following scale: a score indicates that at least 10 cells were found at a particular location; isolated single cells were not recorded.

\+ short migration, not exceeding 10–20 cell lengths (< 200 μm).

++ migration to a distance approximately equal to the dimensions of the graft at the end of the experiment (200–500 μm).

+++ long migration, to a considerable distance from the graft (> 500 μm).

Experimental series 2

The experimental procedure and method of analysis is as for series 1, but, as indicated in Fig. 1B and C, after the graft is inserted, the apex of the limb bud,

i.e. the apical ectodermal ridge (AER) and underlying mesenchyme, is removed. The excision was made in one or other of two ways: in the first (Fig. 1B), the apex of the limb bud was amputated in a straight line across the proximodistal axis. This has the disadvantage of removing a significant part of the mesenchyme and of sometimes leaving in place the anterior and posterior extremities of the AER. In the second (Fig. 1C), the AER and its immediately underlying mesenchyme was removed by two cuts made at an angle to each other, eliminating all of the AER and leaving in place as much as possible of the mesenchyme.

Experimental series 3

The experimental procedure is illustrated in Fig. 5. Fragments of quail wing bud stage 25 (H.H.) were taken for grafting from particular regions of the bud, which was divided into three vertical strips – (1) anterior, (2) central and (3) posterior, and five horizontal strips – from apex to limb base (a–e). The quail limb fragments were grafted into wing buds of fowl embryos stage 23–25 and the material fixed 2 days after the operation. The method of analysis was as for series 1 and 2.

Experimental series 4

In this series, somitic or presomitic tissue containing premyogenic cells at a stage prior to their migration into the limb bud was used for transplantation, as shown in Fig. 6. Strips of somites or unsegmented presomitic mesenchyme were taken at various levels corresponding to the wing, flank and leg along the body axis from quail embryos (stage 13–18 H.H.). Each strip was introduced into a hole made with the emporte-pièce at the base of the fowl host wing bud, stage 18–26 (H.H.). In some cases a fragment of adjoining neural tube was included with the graft. The embryos were fixed 2–5 days later, and analysed as in the previous series.

Experimental series 5

In this series grafts of apical and basal levels of the quail limb bud of quail embryos were made into the coelom of a fowl host embryo as in Fig. 8.

Series 5A. Grafts of apical fragments

Apical fragments of wing buds were taken from quail embryos of stage 21–29 (H.H.). The dimension of these fragments along the proximodistal axis was measured using an eyepiece micrometer and related as a percentage to the total lengths of the limb. For technical reasons, the older the wing bud, the smaller the fragment could be made relative to the whole limb; their order of size related to age is as follows:–

stage 21, 22, 23 – from apex, 15–75 % of limb
stage 24, 25, 26 – from apex, 10–50 % of limb
stage 28, 29 – from apex, 5–55 % of limb

These apical fragments were grafted into the coelom of host embryos of $2\frac{1}{2}$–3 days, placed in such a way that a relation could be established between the graft and the muscular body wall of the host, and fixed 11–12 days after the operation.

Series 5B. Grafts of proximal fragments

In this series the apex of the wing bud was removed and three slices taken from the remaining part of bud in positions 2, 3 and 4 (Fig. 8). Each of the slices was grafted into the coelom of host fowl embryos in the same way as in Series 5A, and fixed 11 days later.

In both series the graft and neighbouring host tissue were sectioned and stained as in the previous series, but in this case scored for presence or absence of host cells in the graft tissues, and for graft cells in the host.

RESULTS

Experimental series 1. Migration of grafted cells in the host limb

The grafts recovered in the sectioned limbs appear as well-integrated masses formed almost entirely of connective tissue and muscle cells, of approximately the same size as the original grafted fragment (300–500 μm). Cartilage appears often and is frequently united with the host cartilage, forming an excrescence on the normal skeleton; or it may form an ectopic island of skeletal tissue. When there is fusion between host and graft tissue the boundary between them is always along a clear line. If the ectoderm of graft and host do not achieve continuity then vesicles may form, extending into the interior of the limb.

Donor cells are also observed in the host tissues beyond the main body of the graft, as in Fig. 2. A small proportion of these cells are found in close connection with the host's nerve axons and we take these to be Schwann cells. But the great majority of them are found, in grafts allowed to develop for 4 days in the host, in the developing muscle masses of the host, and where differentiation has occurred they are clearly incorporated in the myotubes; they must therefore be considered as donor myogenic cells which have moved through the intervening host tissue. Some cells in 4-day cultured and most cells in 2-day cultures are found in the mesenchyme between the graft and the host muscle masses, but never in prechondrogenic areas; we take these to be donor myogenic cells en route to the muscles since at the later stage that is where almost all of them are located.

Direction and extent of cell movement of quail into fowl tissue are indicated in Table 1. All except the shortest movement are in the direction of the limb apex. Some cells of graft origin are found lateral to or behind the graft, sometimes included in host muscle differentiating at the same level as the grafts. Their displacement from the graft never exceeds 15–20 cells and it is difficult to say whether this represents a true movement or a passive displacement caused by (a) the outgrowth of nerve fibres or (b) movement of fowl myogenic cells in an apical direction across the graft, creating a mixed population of cells behind and, even more, lateral to the graft.

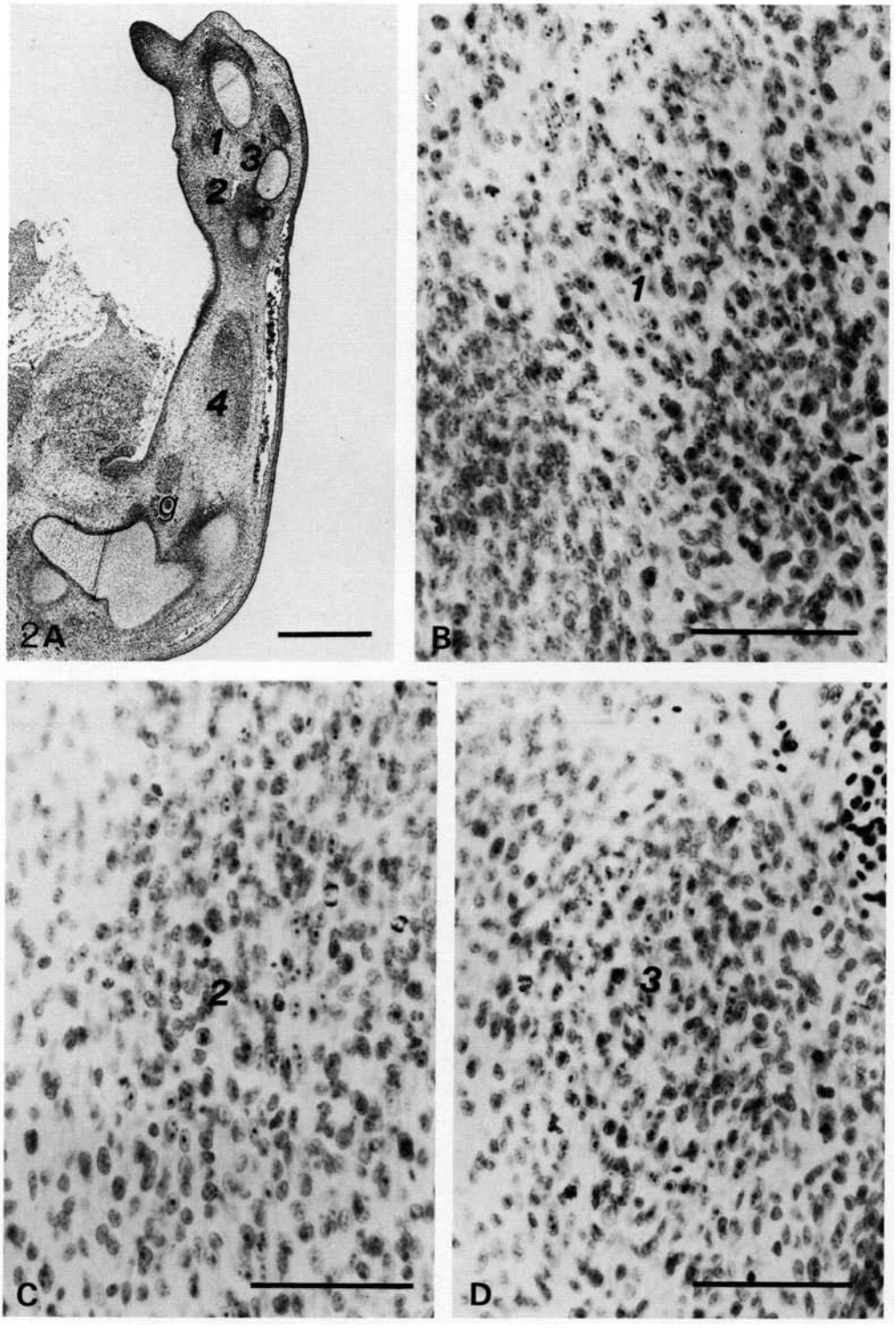
1
3
2
4
g
2A
1
B
2
C
3
D

Table 1. *Emporte-pièce-graft of fragment of quail limb bud into fowl host limb bud (see Fig. 1).*

Experiments	No. of expts.	Cellular movements: Quail → Fowl in direction of limb apex	Cellular movements: Quail → Fowl behind or lateral to graft
Apex present AER present	28	23 (12+++ 10++ 1+)	6 (6+)
Apex amputated (Fig. 1B) with some AER left on stump, and consequent apical development	7	5 (2++ 3+)	2 (2+)
Apex amputated (Fig. 1B) AER absent	10	0	1 (1+)
Apex amputated (Fig. 1C) AER absent	13	5 (5+)	3 (3+)

Explanation in text.

Twenty-nine splanchnic-cell controls were studied and in none was there any indication of movement of grafted cells towards the apex. In the case of undissociated digestive tract fragments, the splanchnopleural musculature does differentiate around the endoderm. On the other hand, quail cells migrating along the nerve fibres – probably Schwann cells – were seen regularly.

In the controls, the graft tissue behaves in the host limb like a foreign body – loose in the case of the young splanchnopleure, compact in the case of the digestive tract, especially in the presence of endoderm. Penetration of host cells into grafts of young splanchnopleure was never observed. In the case of grafts of the older splanchnic mesenchyme there is sometimes a very slight dispersion of the cells, or a very slight penetration of the host cells lateral to the graft (10 cases in all); but in all these cases the phenomenon is extremely limited and if the graft is situated on a pathway of migration of the host myogenic cells those cells move around it.

Fig. 2. Graft taken by emporte-pièce from quail wing bud at stage 24 (H.H.) and grafted into wing bud of fowl embryo stage 20 (H.H.) Fixed 4 days after grafting. (A) general view of implanted wing. g:-level of graft. Bar = 500 μm. (B) muscle marked 2 in (A) – mixed quail-fowl cells. Bar = 50 μm. (C) muscle marked 2 in (A) – mixed quail-fowl cells. Bar = 50 μm. (D) muscle marked 3 in (A) – mixed quail-fowl cells. The muscle marked 4 in (A) similarly contains quail cells mixed with host cells.

Thus, myogenic cells of the splanchnopleure do not migrate into the limb in the direction of the apex, though a few scattered cells from the graft are found in the muscles and skeletal tissue around it. Nor do they provide a pathway of migration for the myogenic cells coming from the somites.

Typical cases are illustrated in Fig. 3.

Experimental series 2. The role of the limb apex in the migration of myogenic cells

In the first type (Fig. 1B; Table 1), 17 cases were studied, fixed at 4 days after grafting. In seven of these, a small part of the AER was left intact and growth of the limb was not arrested so completely as when it was completely removed, in five of these cases there was a small to moderate migration towards the limb apex. It is well known that where the whole AER is removed, only those regions which have already been determined continue to grow so that the limb is shortened and

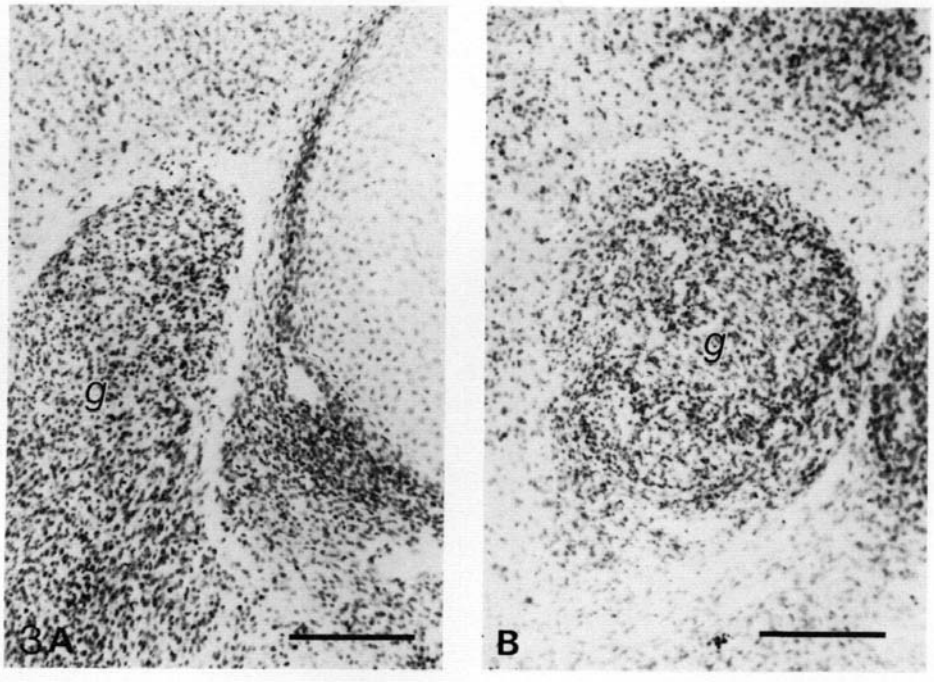

Fig. 3. Controls: grafts of developing gut mesoderm. g: graft. (A) Grafted at stage 22. Graft (g) – mesoderm of gizzard of 5 d quail, separated from endoderm with collagenase. The graft is situated at the level of the humerus. It is very compact and there is no mixing of cells or evidence of dispersion at its boundary. At a distance from it some quail cells are present on the nerve trunks – probably migrating Schwann cells. There is no migration into muscles distal to the graft. Bar = 100 μm. (B) Grafted at stage 25. Graft (g) – quail intestinal mesoderm of 5 days isolated from endoderm. The graft is a very slight dispersion of cells at the immediate periphery of the graft. No quail cells are found in the muscles of the host. Bar = 100 μm. In both (A) and (B) the graft is not well integrated into the tissues of the host and appears as a foreign body.

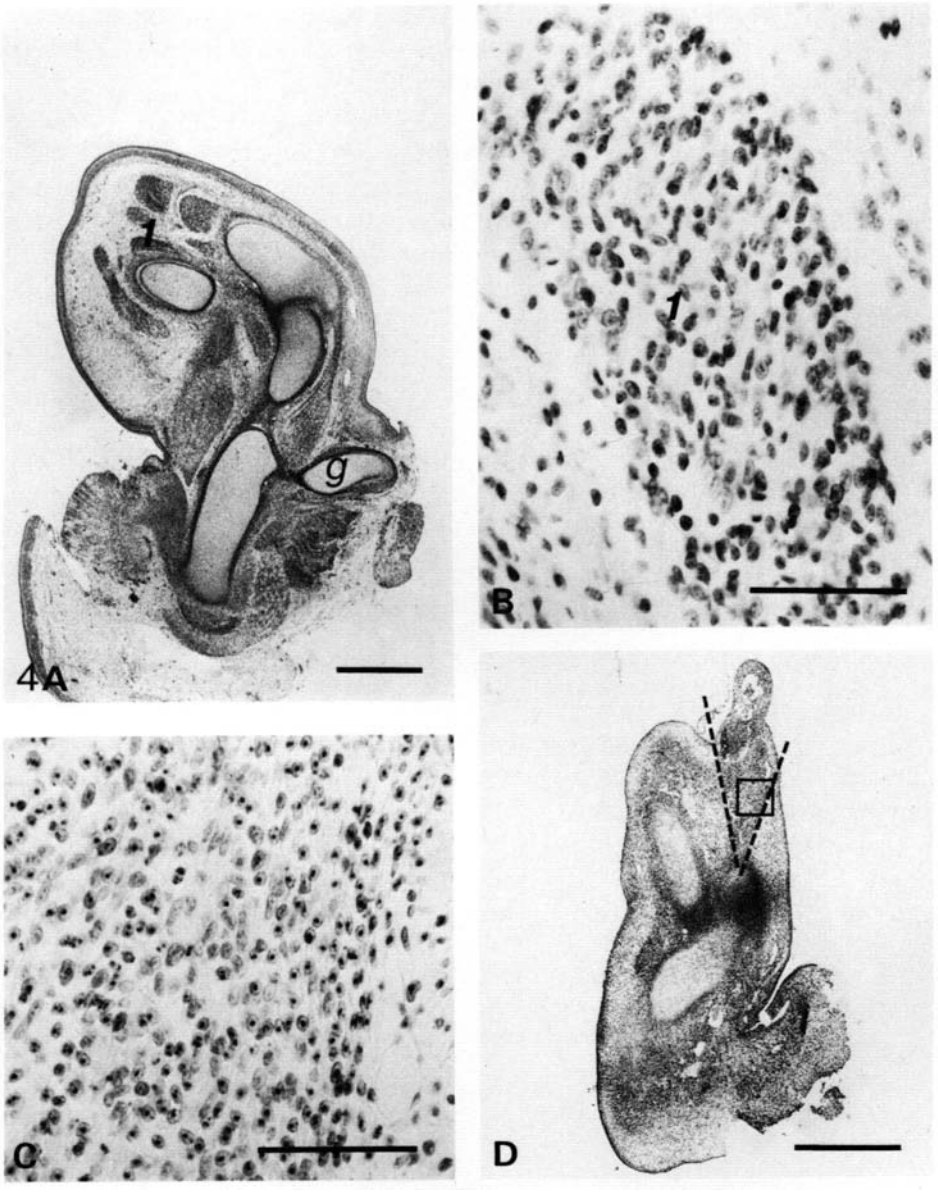

Fig. 4. Emporte-pièce graft quail limb bud fragment into fowl limb bud *in situ*. (A) and (B) as in Fig. 1C. H. stage 20; graft stage 24. (A) General view. g: graft. None of the muscles distal to the graft contain quail cells. Bar = 50 μm. (B) Higher magnification (detail) of the muscle marked 1 in (A). (C) and (D) illustrate one of the special cases produced after operating as in Fig. 1B, in which some quail AER has remained on the graft, leading to outgrowth at this point. Host stage 22; graft stage 22. The graft has produced a second axis of limb outgrowth (indicated by dotted lines). In (C) the region marked by the rectangle in (D) is shown at higher magnification and shows that fowl cells have migrated into the quail tissue in the direction of the AER. In C, bar = 50 μm. In D, bar = 500 μm.

apical elements are not produced. This is what occurred in the other ten cases, examined 2 days after the operation, and in these cases no cell migration in the direction of the apex was observed at all.

In the second type (Fig. 1C; Table 1) 13 cases were studied, fixed 4 days after operation (Fig. 4A,B). The host limb produced a complete or only slightly truncated zeugopod (= radial and ulnar region) and the graft undergoes a proportional growth. The muscles are differentiated in the region between the graft and the apex of the limb, but only from the host cells which, presumably, had already undergone their migration. In five cases some quail cells were observed distal to the graft, but only over short distances and never in the terminal muscles. The presence of quail cells a short distance proximal or lateral to the graft occurs in about the same proportion of cases whether the AER be present or absent.

In seven cases (Table 1), a part of the quail AER was included in a graft which was introduced at the distal edge of the fowl limb bud. Usually a second axis of limb outgrowth is produced, and in these cases numerous fowl cells (probably premyogenic cells) are found in this new outgrowth, presumably migrating towards the supplementary AER. One of these cases is illustrated in Fig. 4C,D.

Experimental series 3. Position of the migrating premyogenic cells in the donor limb bud

Mauger & Kieny (1980) have shown that if material from a fairly late embryonic limb is grafted in place of somites situated opposite to a limb, cells migrate from it into the host limb territory and participate in forming its musculature. We asked if this same material grafted into the limb bud would be capable of migration there. At this stage of development (4½–5 days incubation) myocytes have begun to differentiate at the base of the limb (Grim, 1970); myotubules will

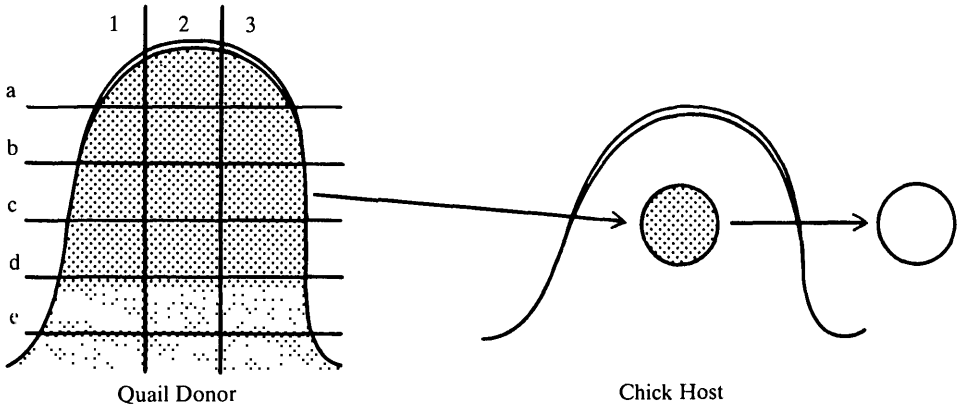

Fig. 5. Fragments of quail wing bud (stage 25) made by dissecting with steel needles and grafted into fowl wing buds at stage 23–25 *in situ*. Fixed 2 days after operation. Further explanation in text.

appear from stage 26. The last premyogenic cells have left somite 20 a little before the stage of 36 somite pairs – at about 3 days of incubation (Chevallier, 1978). At the time of these experiments then, immigration of cells from the somites has stopped at least 2 days previously. Nineteen cases were studied and the results are shown in Table 2.

No difference appears between the behaviour of distal (a,b,c) and proximal (d,e) grafts; migration of premyogenic cells occurs from both, though it occurs to a slightly lesser extent and over shorter distances from the proximal grafts. The important result of this series of experiments is the demonstration that as late as stage 25, when myogenesis has begun at the base of the limb bud, there exists in

Table 2. *Results of grafts of regional fragments of quail limb bud implanted in fowl limb bud as in Fig. 5.*

No. of expts.	Horizontal origin of grafts	Vertical origin of grafts	Cellular movements: Quail→Fowl towards apex	Cellular movements: Quail→Fowl towards base
1	a	1	+++	–
5	b	2	+++	+
		2	+++	–
		2	+	–
		3	+++	–
		3	+++	–
4	c	1	+++	–
		1	+++	–
		2	++	++
		3	++	–
Total 10	a, b, c	1, 2, 3	10 (7+++, 2+++, 1+)	2 (1++, 1+)
3	d	1	+++	++
		2	+++	–
		3	–	–
6	e	1	++	–
		1	++	–
		2	+	–
		2	+	–
		3	+++	–
		3	+++	++
Total 9	d, e	1, 2, 3	8 (4+++, 2+++, 2+)	2 (2++)

Further explanation in text.

this basal region cells which are either in movement or capable of being set in movement when they are placed in a limb environment which is younger and more distal. Comparable results using different techniques have been reported by Chevallier & Kieny (1982).

Experimental series 4. Grafts of somitic material into the wing bud

The somitic cells which form the musculature of the wing begin to leave the somites opposite the wing at the stage when 19–22 somite pairs are present (st. 13–14 H.H.) (Christ *et al.* 1977; Chevallier, 1978). At this stage the wing area is still flat and no AER exists. Most of the somite cells at the level of the wing, when they have the capacity to migrate, can leave the somites and penetrate into the territory of the flank (Gumpel-Pinot, 1974). One cannot therefore invoke the influence of the apex of the limb as inducing the cells to leave the somites. However, one may ask whether somitic cells which are destined to form the wing musculature, placed in a limb environment, will be responsive to the 'attraction' exerted by the apex, responding by leaving the somites and undergoing a polarized migration. This experimental series was designed to answer this question. The strips of somites or unsegmented presomitic mesenchyme were taken from quail embryos at various levels corresponding to the wing, flank and leg along the body axis, but in fact, as regards the myotome derivatives the somitic mesoderm is not regionally differentiated (Chevallier *et al.* 1977). It is known that the last cells to leave somite 20 (posterior wing level) do so a little after the 36-somite-pair stage (Chevallier,

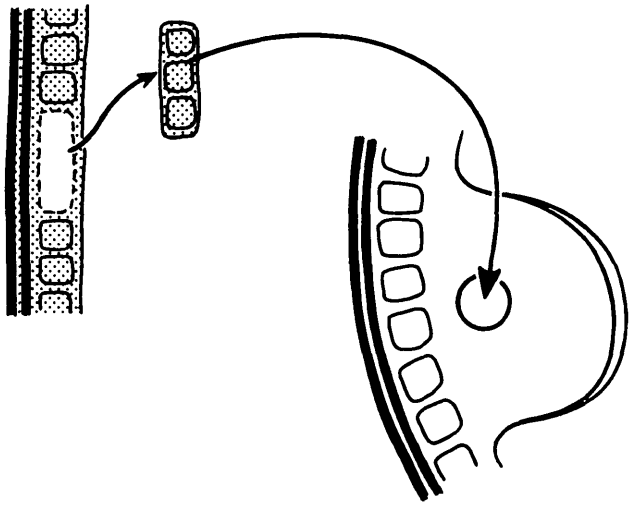

Fig. 6. Strips of somites or presomitic mesoderm from quail embryos (stage 13–18) grafted into wing buds of fowl host embryos (stage 18–26).

1978), so that the somitic or presomitic mesenchyme which we use must contain all or a part of the premyogenic cell population which have the capacity to migrate. Sixty cases were studied. The behaviour of the grafts in the host limb was basically similar to that in Expt. A. Unsegmented presomitic mesenchyme undergoes its segmentation within the limb tissue (Fig. 7), though in this case the development of the somitic material is delayed. In eight cases only, cartilage was formed; probably four representing vertebral cartilage induced by the presence of neural tube; in four cases, cartilage was formed from flank-level mesenchyme with no neural tube, and this probably represents costal cartilage since the ribs are formed in grafts in the absence of neural tube or vertebral cartilage material (Pinot, 1979). The graft sometimes contains mesonephric material and in this case a mesonephric tube may come to open at the surface of the limb, replacing the limb ectoderm at this point.

Migration of myogenic cells from the graft in the direction of the limb apex was observed in only 4/60 cases, and the migration was short (type + or ++); these cases were from grafts taken from embryos with 28 to 34 somite pairs. In 21 cases myogenic cells from the graft were found in the muscles of the host immediately surrounding the graft – here again, it is difficult to say whether this represents

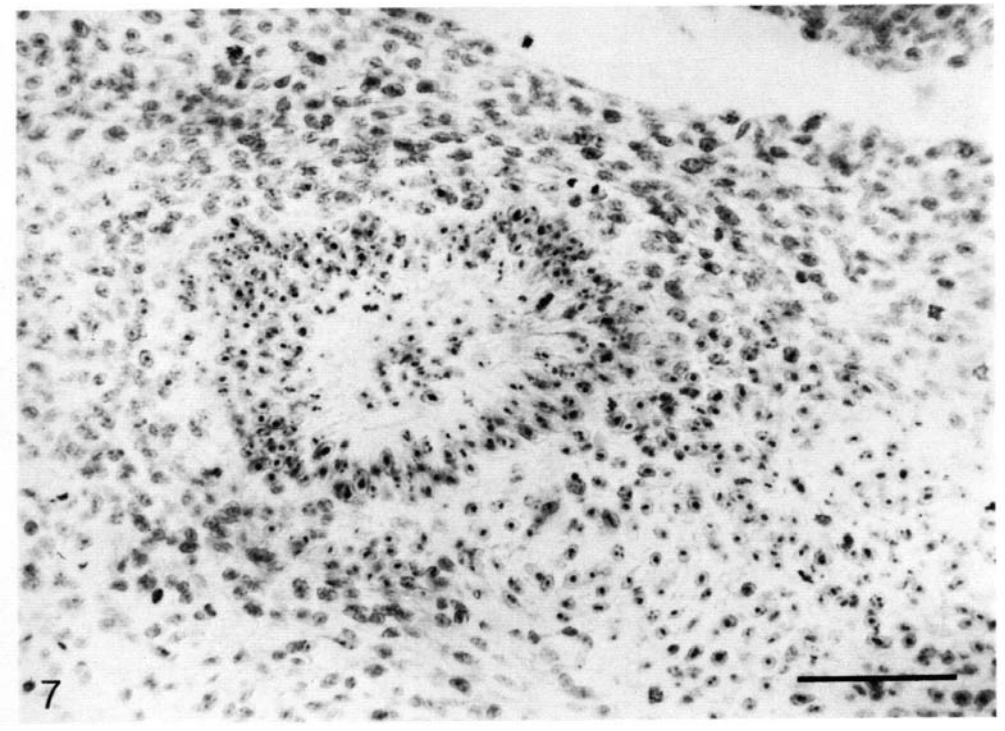

Fig. 7. Strip of presomitic mesoderm from quail (28 somite-pairs) taken from leg level, grafted into fowl limb bud stage 24. The graft has remained localized. No migration into distal muscular formations is observed. Note that, in contrast to grafts in Fig. 3, the graft has been well integrated into the host tissues. Bar = 50 μm.

true movement of the quail cells, or of a dispersion of them by the migration of host myogenic cells across the graft. Some quail cells are found at a long distance from the graft on the nerve fibres, and these are probably ganglion or Schwann cells.

The premyogenic cells contained in the somites and destined under normal circumstances to migrate into the limb rudiment do not seem to be capable of moving out from the somite or migrating in the direction of the limb apex in the conditions of this experiment. It may be that they are not responsive at this stage to the polarization created by the apex, or that the particular conditions – e.g. of extracellular matrix structure – required for their emigration from the somite do not exist in the limb bud.

Experimental series 5. Coelomic grafts taken from apical and basal levels of the wing bud

One possibility which the above results suggest is that there might be a gradient of some sort within the developing wing, dependent on the limb apex, and controlling the orientation and migration of muscle cells of somitic origin. If this were the case, an apical fragment of the limb bud grafted into the coelom in such a way as to develop in close relation with the body wall of the host where the myogenic cells are moving into place should 'attract' these myogenic cells to itself and this attraction might well be stronger in more apical limb bud fragments if they are from younger donor limbs. These experiments test this possibility.

Series 5A. Wing apex grafts. Forty grafts were studied. Each graft forms a mass of tissue, rounded or lengthened, surrounded by its well-developed feather germs. They are either free in the coelomic cavity, attached to the host by only a connective tissue stalk with blood vessels, or, as we wanted it to be, fused to

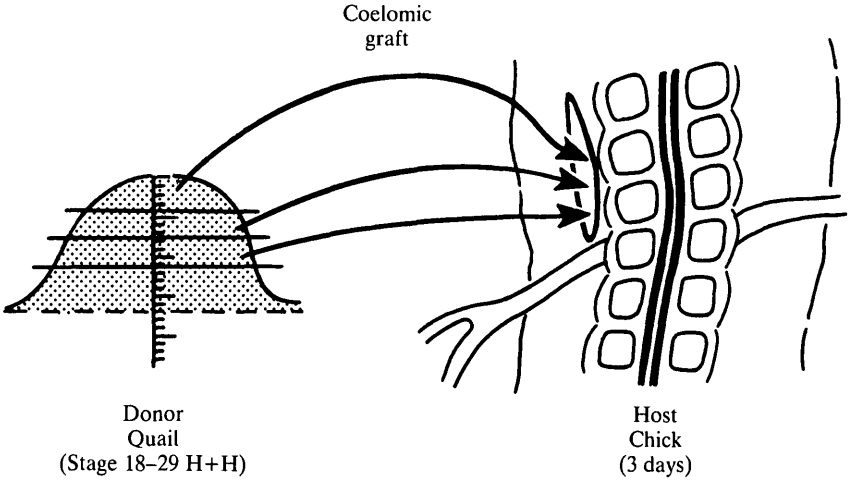

Fig. 8. Grafts of apical limb bud fragments from quail embryos (stage 18–29) grafted into coelom of fowl host embryos at 3 days. Fixed 11–12 days after operation.

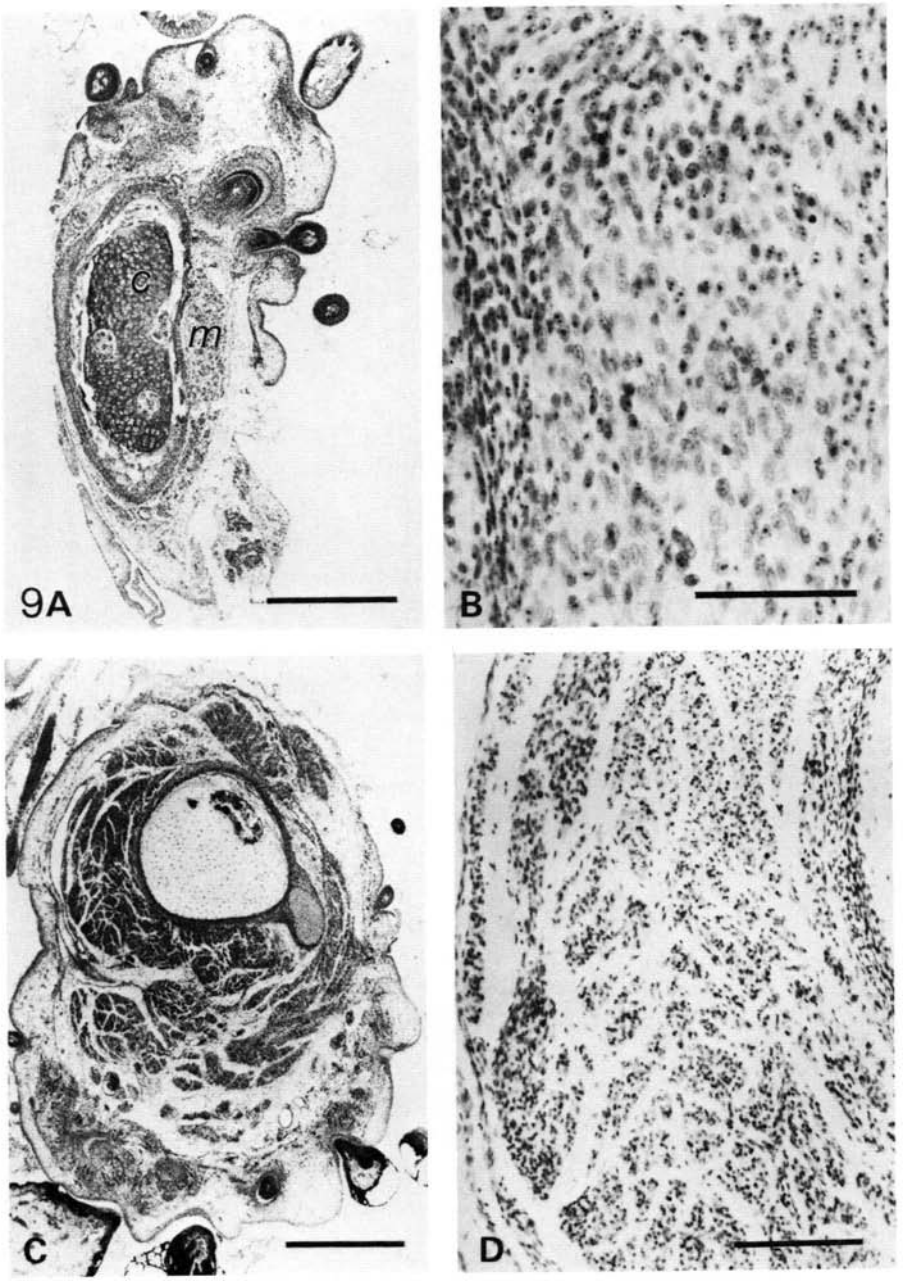

Fig. 9. Results of coelomic grafts of apical limb fragments (A) and (B) as in Table 3, Series A and proximal slices (C) and (D) as in Table 3, Series B. (A) Graft of apical 24 % of quail wing bud (stage 24). Fixed 10 days after grafting. General view. Graft is small with central core of cartilage – (c), and a very small muscle formation – (m) – which is of mixed quail/fowl cells. Bar = 500 μm. (B) Detail of muscle in graft (A), showing mixed quail/fowl cells. (D) Graft of a proximal slice representing about 25 % of a quail wing bud (stage 24). Fixed 10 days after grafting. General view, showing cartilage and extensive muscle formation. Bar = 100 μm. (C) Detail of muscle in (D) showing that the muscle consists exclusively of quail cells. Bar = 500 μm.

Table 3. *Coelomic grafts of apical (series A) and proximal (series B) segments of limb buds (see Fig. 3)*

Graft	No. of expts.	Graft attached to coelom wall of host	Migration Host → graft	Migration Graft → host
Apical 1 (series A)	40	16	11	0
Proximal 2, 3, 4 (series B)	12	11	0	2

the body wall. Parts of the skeleton were present in 39 of the grafts, their degree of development varying, according to the size of the graft and its stage, from one small nodule to two complete elements.

Muscle fibres and organized skeletal muscles of graft origin are recognizable in 37 cases (none of the three negative cases corresponds to the smallest fraction grafted for a given stage); the muscles of the feathers are well developed and are also of graft origin.

In 16 cases the graft is fused to the body wall of the host by its base, its apex pointing into the coelom. In 11 of these cases (Table 3) there are in the anterior of the graft some muscles of mixed graft (quail) and host (fowl) origin (Fig. 9A). Myogenic cells of the host have moved into the graft where they participate in its own developing musculature (Fig. 9B). These 11 grafts were taken from donor embryos of stage 22–28 (H.H.); the smallest fragment represents 18 % of the limb (stage 25), the largest 55 % (stage 28). By contrast, no myogenic cells of the quail are found in the body wall of the host.

The grafts which are attached to the body wall of the host by a connective tissue stalk frequently show host cells at their base, but these seem to be only connective tissue cells, not participating in any of these cases in the musculature. The same phenomenon is found in coelomic grafts of other tissues – e.g. meso- or metanephros, or heart (Gumpel-Pinot, unpublished observations).

In summary, where an apical fraction of the wing bud of stage 22–28, whatever its size, is present, myogenic cells of the host migrate into it, provided that the tissues of the host and the graft are in intimate contact.

Series 5B. Grafts of proximal fragments. Twelve grafts have been studied (Table 3). In 11 cases the graft become attached to and even embedded in the muscular body wall of the host, and developed cartilaginous and muscular structures (Fig. 9C). In no case were any myogenic cells from the host observed in the graft (Fig. 9D). In one case there was a slight dispersion of graft cells in the muscles of the flank. In another case the graft was retrieved at the base of the host wing and here there was a clear migration of graft cells towards the apex of the wing.

From these experiments one may conclude that these regions of the wing bud (even if they are probably not – as in the case of position 2 – completely colonized by their own myogenic cells) are not invaded by the myogenic cells of the host, and that only the limb apex is so invaded. This suggests that there is no P–D gradient within the limb bud which controls myogenic cell movement.

DISCUSSION

Three striking findings emerge from these results: (a) migration of implanted myogenic cells occurs within the developing wing bud; (2) this migration is orientated towards the bud apex; (3) it does not occur in the absence of the apical ectodermal ridge (AER).

The studies of Gumpel-Pinot (1974), Chevallier (1978) and Jacob, Christ & Jacob (1978 and 1979) have established that cells leave the somites from stage 14, moving into the somatopleural presumptive limb region while it is still flat, becoming localized close to the coelomic epiblast. When, at stage 18, the wing bud is established as a distinct swelling with an AER these myogenic cells become distributed through the limb mesoderm. In the course of further development they become arranged as differentiating myoblasts of the definitive muscle pattern within the limb; within the muscle masses the myogenic cells sort out from the somatopleural cells which form the supporting connective tissue.

Our experiments, and those of Wachtler, Christ & Jacob (1982) show that these myogenic cells undergo a further distalward migration when implanted into the limb bud. Wachtler *et al.* (1982) show that somatopleural without somitic tissue gives no migration at all, and we show that splanchnopleural tissue, even though it contains myogenic cells of its own type, also gives no migration. The capacity of myogenic cells to migrate after forming muscle rudiments has been investigated by Mauger & Kieny (1980), who showed that myogenic cells from 4- to 5-day quail premuscular masses are able to undergo extensive migration into the limb buds when substituted for somites in early embryos.

Studies by Newman (1977) and Hauschka & Rutz (1983) in which cells from different proximodistal levels have been cultured *in vitro* to give recognizable cell types have shown there to be a gradient of myogenic cells within the limb bud, consistent with a colonization of the apical limb mesenchyme by invasion from more proximal regions. It appears that as the limb bud grows out, myogenic cells of somitic origin progressively invade more distal regions, and that this capacity for myogenic cell migration may be continued or resumed under experimental condition.

Migration in intact wing buds

There is a zone of mesenchyme beneath the AER, of the order of 500 μm deep, which from the *in vitro* culture experiments referred to above appears to contain no myogenic cells. We propose that as the limb bud grows out, myogenic

cells from more proximal regions advance into the proximal border of this zone and colonize it, probably becoming subject then to the pattern-determining mechanisms – diffusing ZPA-morphogens, polar-coordinate cell-interactions, or whatever it may be – which operate there. Ahead of this colonized region, the myogenic cell-free zone is renewed by cell division as limb outgrowth continues.

In our experiments, where quail cells are grafted into a limb bud whose apex is intact, myogenic cells resume their capacity for migration and move distalward into the cell-free zone. Quail myogenic cells are shown to be invasive with respect to chick limb mesenchyme, and in some cases chick myogenic cells invade quail limb mesenchyme; we conclude further that limb myogenic cells are invasive in limb mesenchyme in normal avian development. Tickle, Goodman & Wolpert (1978) reported that when stage-22 quail limb mesenchyme was grafted into stage-21 chick wing no quail cells migrated into the chick mesenchyme. This contradiction remains to be explored, but Tickle (1982) has reported that cells which are known to be of highly invasive type – mouse trophoblast cells, macrophages, transformed cells – do so migrate. We must conclude that limb myogenic cells belong to this invasive category. Like many other invasive cells – e.g. neural crest cells, melanocytes, sarcoma cells – the limb myoblast cells synthesize little fibronectin (Chiquet, Eppenburger & Turner, 1981).

The mesoderm of the limb bud consists of a fairly loose arrangement of mesenchyme cells with considerable intercellular space between, filled with extracellular matrix. Excepting where condensations of cells has occurred, e.g. in the proximal cartilage core from an early stage, there is sufficient space for cells to send out filopodia between cells without hindrance, and for actual movement of the main cell body to occur with some pulling, pushing and shoving. What factors – presumably in the ECM – orientate the myogenic cells in their migration can only be conjectured at present. Fibronectin is much canvassed as a candidate for directing the movement of cells in morphogenesis, particularly where the cells involved do not synthesize much themselves. Melnick *et al.* (1981); Kosher, Walker & Ledger (1982) and Tomasek, Mazurkiewicz & Newman (1982) have found various nonuniformities in FN-distribution in the developing chick limb, though hardly well-defined tracks.

Whether or not tracks of any sort exist, the exclusively proximodistal direction of myogenic cell migration remains to be explained. We propose that the myogenic cells, being invasive relative to the somatopleural limb mesenchyme cells, will tend to move within the mesenchyme wherever resistance is least. There is good reason to suppose (see discussion in Ede, Bellairs & Bancroft, 1974) that in the developing limb bud mesenchymal cells are steadily shuffling distalwards, producing the elongation of the limb bud in the absence of any sufficient localized control of mitosis; it may be autonomous to the mesenchyme cells, or imposed by proliferation within a constraining ectodermal membrane. Either way, the cells are moving slowly in an apical direction, so that invasive cells forcing their way between them will be in the same position as runners

wanting to push their way through a crowd of slow walkers – i.e. they will find it much easier to run through the crowd in the direction it is taking, rather than force their way through against it. In this way the distalward direction may well be imposed on the migrating myogenic cells.

The effect of removing the apical ectodermal ridge on growth and differentiation was described by Saunders (1948); proximal regions continued to grow and differentiate, but more distal – as yet undetermined – regions did not, so that only the stump of a limb was produced.

Our experiments show that in the absence of the AER, together with a minimal amount of dorsal and ventral ectoderm and underlying mesenchyme, migration of the implanted myogenic cells does not occur. Using an *in vitro* assay technique, Hauschka & Rutz (1983) have found that where the AER is absent, myogenic cells cannot be found in the distal regions of the limb bud. Presence of the AER appears to be a necessary condition of the distalward migration of the myogenic cells. In our experiments, chick myogenic cells were certainly found at the tip of the resulting limb stumps, and these cells had presumably migrated into the colonizable zone – the presumptive material of the stump tip – before the removal of the limb apex. But quail cells were in some way inhibited from moving beyond the graft by this operation.

The apical ectodermal ridge has properties which distinguish it from the dorsal and ventral limb ectoderm on either side of it. Fibronectin is particularly abundant subjacent to its basal lamina (Tomasek *et al.* 1982), and this may make it a site of preferential attachment for cell processes of the limb mesenchymal cells in its neighbourhood. Ede, *et al.* (1974) have described the long filopodial extensions from mesoderm cells which connect those with the AER, sometimes from several cell distances away, and it is possible that these cells align others behind them, leading to the production of an orientated ECM through the limb bud, in much the same way as Fitzharris & Markwald (1982) have suggested that filopodial probing by endocardial cells and pioneering cells results in macromolecular re-orderings of the ECM in the developing heart. Removal of the limb-bud apex would disrupt this connection and the ECM guide lines arising from it, and this might in itself cause the failure of myogenic cell migration in the absence of the AER.

Furthermore, Kaprio (1979) has shown *in vivo* and Kosher, Savage & Chan (1979) *in vitro* than when the AER is removed the cells of the subridge mesoderm become very closely packed, with large areas of intimate surface contact between adjacent cells and little if any intercellular space. Thus the effect of removing the AER may be simply to make the colonizable zone impenetrable by the normally invasive myogenic cells, or by the filopodial extensions of these cells. The zone will already have been colonized by the host myogenic cells, so that in these experiments the muscles distal to the graft in the limb stumps will be exclusively of host origin. An alternative view would be to suppose that the presence of the AER in intact limb buds normally excludes myogenic cells from entering the

apical mesenchyme, but the result of our experiments of series 5 suggest that this is not the case, since host cells do enter the grafted wing apex but not more proximal regions. There are of course other possibilities to be considered involving interaction at a distance between the AER and the responding myogenic cells.

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