

## Tissue and cellular patterning of the musculature in chick wings

L. G. Robson\*, T. Kara, A. Crawley and C. Tickle

Department of Anatomy and Developmental Biology, University College Middlesex School of Medicine, Windeyer Building, Cleveland Street, London W1 6DB, UK

\*Author for correspondence at present address: MRC Muscle and Cell Motility Unit, The Randall Institute, 26-29 Drury Lane, King's College London, London WC2B 5RL, UK

### SUMMARY

Development of the musculature involves generation of a precise number of individual muscles arranged in appropriate locations, each with the correct cellular patterning. To find out the rules that govern muscle number and arrangement, the forearm musculature of chick wing buds was analysed following grafts of the polarizing region or application of retinoic acid. Muscle patterns appear symmetrical with 'posterior' muscles now forming in the anterior part of the wing. When the number of muscles that develop is reduced, pattern symmetry is maintained, with loss of anterior muscles in the mid-line, especially dorsally. Strict anteroposterior ordering of muscles in duplicated patterns does not always occur. The number of muscles that develops bears some relationship to the number of forearm elements. Each muscle has a characteristic pattern of fast and slow fibres. In duplicated wings, each pair of symmetrically arranged muscles has the same fibre type

pattern. Not only are proportions of fast and slow fibres similar, but local variations in fibre type arrangement within the muscle are also reproduced. This suggests that the cellular pattern within the new 'posterior' muscles at the anterior of the limb has been re-specified. In manipulated limb buds, which will develop a duplicated muscle pattern, there are no detectable changes in distribution and number of potentially myogenic cells, and fibre type patterning within early muscle masses also appears normal. In contrast, the splitting process that divides up muscle masses is altered. The appropriate fibre type arrangement only emerges after splitting is complete. This suggests that tissue patterning and cellular patterning occur at different times during muscle development.

Key words: chick limb, retinoic acid, muscle fibre type

### INTRODUCTION

A major question in development is how precursor cells of various tissue types become organised and arranged in the proper places. In the developing limb, myogenic precursor cells undergo patterning, at both tissue and cellular levels, to give rise to the precise arrangement of specific muscles and the precise arrangement of fibre types within each muscle. A separate but related problem is what determines muscle number. Manipulations of developing chick wings lead to changes in tissue patterning that have been particularly well analyzed with respect to the skeleton. However, the pattern of muscles can also be changed and here we explore the basis of tissue and cellular patterning of muscle by manipulating the wing bud of chick embryos.

The muscles of the limb are composed of myogenic cells that are derived from the lateral half of the somites, whereas the muscle sheaths are derived from connective tissue cells of the lateral plate mesoderm (Ordahl and Le Douarin, 1992; Kenny-Mobbs, 1985). In early limb buds, potentially myogenic cells congregate into dorsal and ventral muscle masses (Schramm and Solursh, 1990). Individual muscles are formed by divisions of these muscle masses (Shellswell and Wolpert, 1977). Myogenic cells differentiate and fuse to form muscle

fibres, which synthesize either fast or slow muscle proteins (Sweeny et al., 1989; Hilfer et al., 1973).

The pattern of the musculature appears to be controlled by the connective tissue (Chevallier and Kieny, 1982). For example, when pieces of unsegmented thoracic mesoderm are transplanted in place of brachial somites, the donor myogenic cells form an appropriate brachial musculature (Butler et al., 1988). The cellular pattern of fast and slow fibres within individual muscles is also reproduced (Butler et al., 1988) suggesting that the connective tissue in some way governs both tissue and cellular patterning.

The potentially myogenic cells migrate into the limb forming region of the embryo before the limb develops (Jacob et al., 1978; Solursh et al., 1987). Furthermore, when cells from early limb buds are placed in high density culture, myogenic cells differentiate (Schramm and Solursh, 1990). Even in early limb buds, there are distinct populations of potentially myogenic cells that can be distinguished by their expression of particular developmental forms of the myosin heavy chain (MyHC) in culture (Miller and Stockdale, 1986; Crow and Stockdale, 1986).

It is not known how the 13 muscles of the forearm region of the chick wing are assembled from the potentially myogenic cell population. In the development of the musculature of the

lower jaw, which is not so complex, the cellular pattern appears to be laid down by precursor cells very early in development, and parcelling of potentially myogenic cells automatically leads to the muscle fibre type pattern.

Manipulations of the chick wing produce skeletal pattern changes (reviewed by Tickle, 1991). The musculature is also affected (Shellswell and Wolpert, 1977) and this provides a way of analysing the mechanisms involved in tissue and cellular patterning of muscles. The pattern of structures across the antero-posterior axis of the wing bud appears to be controlled by a signal from the polarizing region, a small group of cells at the posterior margin of the bud (Saunders and Gasseling, 1968; Tickle et al., 1975). When the polarizing region from one wing bud is grafted to the anterior margin of a second wing bud, anterior cells are respecified to form posterior structures. For example, 6 digits develop instead of 3, and an additional set of 432 develops in mirror image to the normal set of 234. The change in pattern can affect cells of the connective tissue lineage in the forearm and a duplicated mirror-image set of muscles develop associated with 2 ulnae (Shellswell and Wolpert, 1977). The signal from the polarizing region can be reproduced by local application of retinoic acid (Tickle et al., 1982), and both skeletal and muscle patterns are duplicated (reviewed by Brickell and Tickle, 1989). Here we manipulated limb buds either by a polarizing region grafts or applying retinoic acid and explore both the tissue and cellular muscle patterns. We also investigate the way in which muscles are constructed in both normal and manipulated limb buds.

## METHODS

### Manipulations to alter anteroposterior pattern of chick wing buds

Polarizing regions from stage-20 to -21 chick limb buds (Hamburger and Hamilton, 1951) were grafted to the anterior margin of stage-18 to -20 chick wing buds. The manipulation causes duplication of limb structures distal to the humerus. The polarizing regions were dissected from donor limb buds by first removing the ectoderm using 2% trypsin at 4°C for 1 hour. The polarizing regions were grafted under a loop made along the anterior portion of the apical ectodermal ridge of host wing buds (Tickle, 1981). In a few cases, polarizing regions were grafted to the apex of the host limb under a loop of the apical ectodermal ridge.

Controlled release carriers, AG1-X2 beads (in formate form, from Bio-Rad), of 200 µm diameter were soaked in a solution of either 10, 1 or 0.1 mg/ml all-*trans*-retinoic acid (Sigma lot 40H0313) in DMSO. After the beads were rinsed in culture medium (minimum essential medium (MEM) + 10% FCS (GIBCO Biocult)), they were implanted, under anterior loops of ridge ectoderm of stage-20 chick embryos (Tickle et al., 1985).

### Histology of manipulated limbs

Embryos were allowed to develop for 6 days after either a polarising region graft or retinoic acid bead implantation. The embryos' wings were fixed in either 5% trichloroacetic acid (TCA) for wax embedding or half strength Karnovsky's fixative for Araldite, for 24 hours. The cartilage elements of the wings were stained with Alcian Green and then the specimen was cleared in methyl salicylate. Limbs were photographed to show the gross anatomy of skeletal elements. To investigate the muscle pattern, limbs were embedded either in wax, and sectioned at 7 µm, and stained with Mallory's trichrome, or in Araldite, sectioned at 1 µm, and stained with Toluidine blue. To check

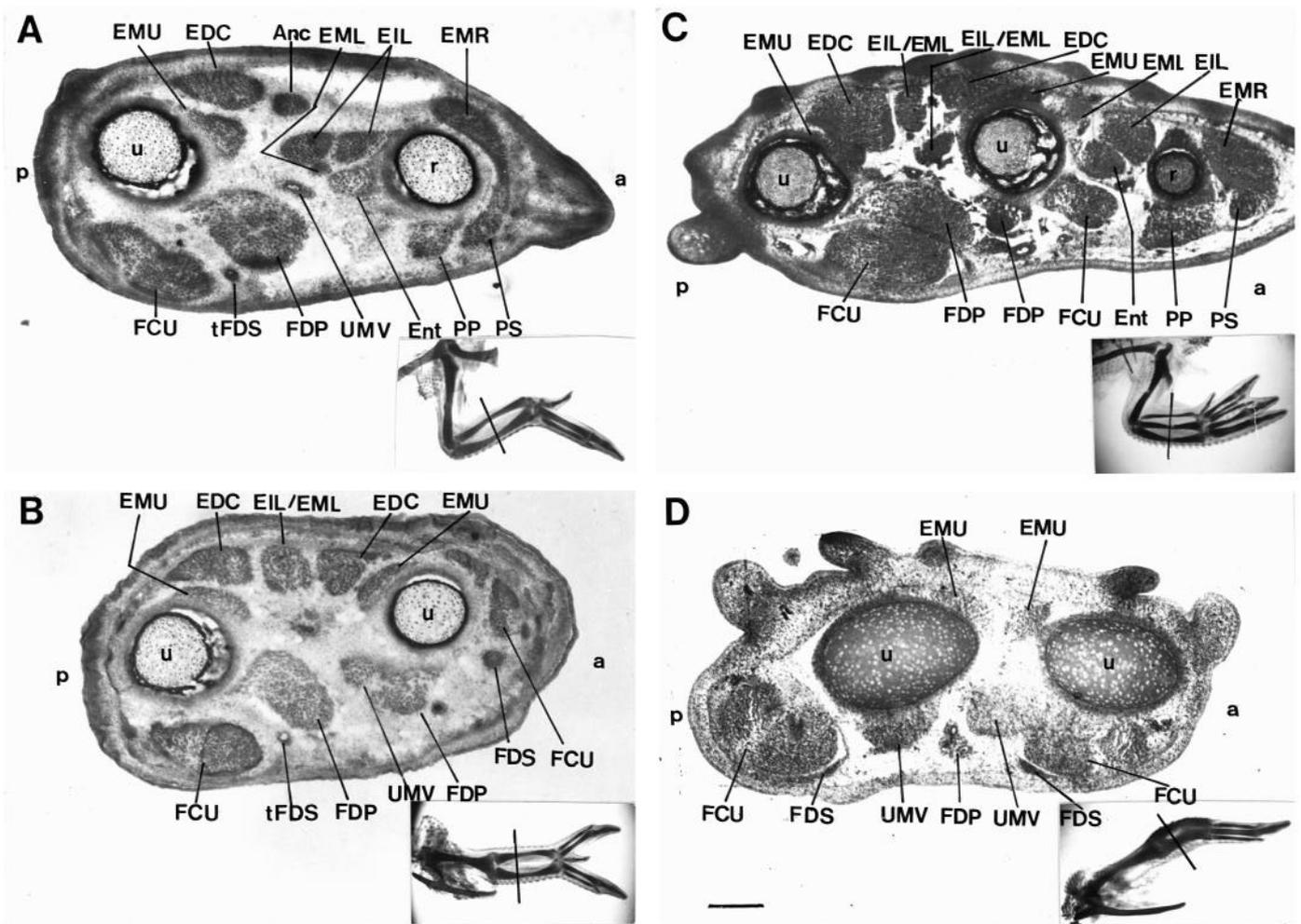
the muscle pattern before immunolabelling, several cryosections from normal and manipulated wings were fixed in 70% alcohol and stained with Mallory's trichrome.

### Muscle identity

Sullivan (1962) and Shellswell and Wolpert (1977) describe the 13 muscles in the chick forearm. Each muscle has a characteristic position, origin and insertion. In transverse sections, muscles can be identified by their location and shape (Shellswell and Wolpert, 1977; Shellswell, 1977) and this identity can be confirmed by tracing individual muscles in serial transverse sections. In transverse sections of the forearm, the dorsal musculature consists of 6 evenly arranged muscles; anterior to posterior: extensor metacarpi radialis (EMR), extensor indicis longus (EIL) and extensor medius longus (EML) associated with the radius; and extensor digitorum communis (EDC), anconeus (Anc) and extensor metacarpi ulnaris (EMU), associated with the ulna (Fig. 1A). EMR, is an arc-shaped muscle that lies over the anterior surface of the radius. EIL lies on the other side of the radius towards the centre of the limb, and its tendon forms a bar across the muscle belly splitting the muscle. EML is a rectangular muscle that lies ventral to EIL. Next, EDC is a large wedge-shaped muscle near the ulna. EMU is an arc-shaped muscle that lies over the inner surface of the ulna and Anc is a small muscle in transverse section that lies central to EDC (Fig. 1A). Ventrally, 7 muscles are arranged asymmetrically across the limb; with more muscles being clustered under the ulna in the posterior part of the limb. From anterior to posterior the muscles are pronator superficialis (PS), pronator profundus (PP) and entepicondylar (Ent), associated with the radius; and flexor digitorum profundus (FDP), ulnometacarpalis ventralis (UMV), flexor digitorum superficialis (FDS) and the flexor carpi ulnaris (FCU) associated with the ulna. PS is an arc-shaped muscle that extends around the ventral anterior surface of the radius; PP is a round muscle in transverse section under the ventral surface of the radius. Ent is a small muscle, central to the radius. These three muscles are seen in this arrangement in transverse sections taken at the mid radius/ulna level. PS and PP insert onto the radius and Ent inserts on the ulna just distal to the mid point of the forearm, and are not seen in more distal sections. FDP is the largest muscle in the forelimb. It lies in the centre of the ventral wing musculature, and is squarish in shape and points towards the ulna. UMV is closely associated with FDP, and is a triangular-shaped muscle in transverse section and lies central to the ulna (in Fig. 1A the section has just passed through a corner of UMV). The smallest muscle of the ventral musculature is FDS which forms an arc of muscle that lies ventral to UMV and FDP. In Fig. 1A only the tendon of FDS has been sectioned, the muscle appears in slightly more distal sections. FCU has a tendon that runs along the dorsal surface that causes an in-tucking of the muscle, creating a hook-shaped larger portion towards the centre of the limb and a smaller rounded profile posteriorly. In many cases, muscle identity could be fairly readily deduced from a single section, but sometimes the suggested identity was deduced by examining adjacent sections both proximally and distally.

### Antibody labelling of limb muscles

The manipulated right and contralateral control left limbs were dissected from embryos 6 days later. The limbs were first dissected into PBS and then taken through a graded series of increasingly strong sucrose solutions (5, 7, 10 and 16 and 20% sucrose solutions in PBS) until they had sunk (approximately 4 hours). The limbs were frozen in a mixture of 20% sucrose and OCT (BDH (2:1)) by immersion in liquid nitrogen and then sectioned at 7 µm on a Reichert 'Frigocut' cryostat, as previously described (Robson, 1993). Sections were reacted with the appropriate mouse monoclonal antibodies to detect the skeletal MyHC. Antibody LM5 was used to detect all developmental forms of the fast MyHC, including the embryonic form, while antibody 98/31 is specific to the slow MyHC-I. Both antibodies were the kind gift of Dr G. K. Dhoot (Dhoot et al., 1988; Dhoot, 1989). A



**Fig. 1.** Pattern of muscles and skeletal elements (insets show typical skeletal pattern obtained and the position of the section). (A) Normal 10-day chick wing. (B) Wing following polarizing region graft to anterior margin. (C) Wing following polarizing region graft to apex of bud. (D) Wing with bead soaked in 10 mg/ml retinoic acid implanted at anterior margin. Experimental wings were fixed 6 days after operation. The normal limb (A) has a full complement of 13 muscles. The limb with an anteriorly grafted polarizing region (B) develops 5 digits (43234); in the forearm, 2 ulnae and 12 muscles. The wing in C has 6 digits (234334) and 3 forearm skeletal elements, a radius and 2 ulnae, and 16 muscles. The limb treated with a bead soaked in 10 mg/ml retinoic acid (D) has fewer muscles (9), 3 digits (434) and two forearm elements. The suggested identification of the muscles is based not only on their appearance in these transverse sections, but also by reference to more proximal and distal sections. Scale bar, 200  $\mu$ m. a, anterior; p, posterior.

fluorescein-conjugated second antibody (Dakopatts) was used to detect antibody binding. Sections were mounted in DABCO (1,4 diazobicyclo-(2,2,2)-octane) to prevent fading of the fluorescent labelling, and photographed.

#### Splitting of the muscle masses

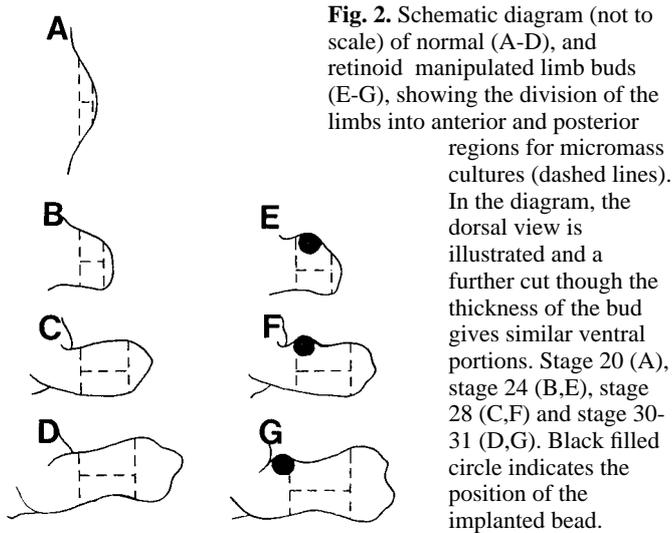
To follow the splitting of muscle masses in manipulated limbs, beads soaked in 0.1 mg/ml retinoic acid were implanted at the anterior margin of chick wings. Both manipulated and control limbs were cryosectioned at 48 hours and 85 hours after bead implantation. The muscle masses were analysed by immunolabelling (see above).

#### Cell culture and immunolabelling of myogenic cells

Micromass cultures were prepared from untreated limb buds of stage-20 embryos, and both retinoid-treated and -untreated limb buds of stage-24, -28 and -30/31 chick embryos. The limbs were dissected out in calcium and magnesium free saline (CMF) + 10% fetal calf serum (FCS). The limbs were divided into dorsal and ventral posterior and anterior regions as shown in Fig. 2. Only the presumptive forearm

regions of the limbs were cultured; the tip and proximal regions of the limb buds were cut away. The ectoderm was removed from the tissue fragment by a cold 2% trypsin treatment, as outlined above. The mesenchyme was then disaggregated in cold CMF + 10% FCS by vigorous pipetting. The cells were spun down at 3000 *g* for 3 minutes, resuspended in 1 ml of CMF + 10% FCS and the number of cells per unit volume calculated, before being spun down again. The cells were resuspended in a 'defined medium'. This defined medium has been shown to increase the differentiation of myoblasts in micromass culture conditions (Robson, 1993). The cells were resuspended to a final volume of  $2 \times 10^5$  cells per 10  $\mu$ l. 10  $\mu$ l drops were placed into 15 mm diameter 4-well tissue culture grade dishes (one drop per well) and incubated at 38°C with 5% CO<sub>2</sub>. After 1 hour, cultures were flooded with 200  $\mu$ l of fresh medium and returned to the incubator. The culture medium was replaced every 24 hours.

Myogenic cells were identified in cultures after 2.5 days using monoclonal antibodies to MyHC's and visualized with a colloidal gold second antibody (Biocell) and silver enhancing as described by Robson (1993).



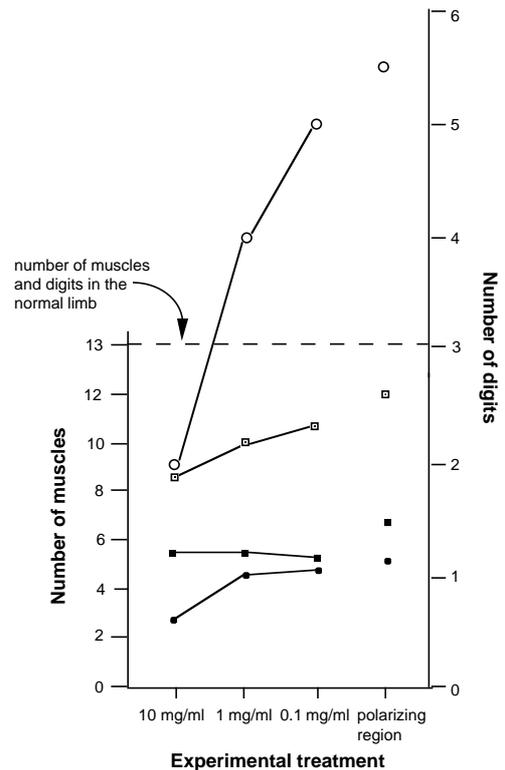
**Fig. 2.** Schematic diagram (not to scale) of normal (A-D), and retinoid manipulated limb buds (E-G), showing the division of the limbs into anterior and posterior regions for micromass cultures (dashed lines). In the diagram, the dorsal view is illustrated and a further cut through the thickness of the bud gives similar ventral portions. Stage 20 (A), stage 24 (B,E), stage 28 (C,F) and stage 30-31 (D,G). Black filled circle indicates the position of the implanted bead.

**RESULTS**

**Muscle patterns of manipulated limbs**

Early wing buds were manipulated by either grafts of polarizing region cells or application of retinoic acid. The effects on muscle number and muscle pattern were assessed. We used criteria outlined in the Methods to identify most of the muscles in the duplicated limbs. Our identifications are indicated in sections shown in Fig. 1, and in later figures.

When either a polarizing region or a bead soaked in 0.1 mg/ml retinoic acid is placed at the anterior margin of an early limb bud, the number of muscles that develop is more or less unchanged. Following polarizing region grafts, 11-13 muscles develop (mean = 12, s.d. 0.6); 5-6 dorsally and 6-7 ventrally (Figs 3, 4). With local application of beads soaked in 0.1 mg/ml retinoic acid, the number of muscles in the forearm was on average 10 (s.d. 1.5), slightly fewer than with the polarising region grafts. With both sets of manipulations, the pattern of muscles appears symmetrical, both dorsally and ventrally. Fig. 1B shows a transverse section through the forearm of a wing following a polarizing region graft and should be compared with Fig. 1A (a transverse section of a normal wing). In the manipulated limb (Fig. 1B), there is a symmetrical muscle pattern. Dorsally, 5 muscles can be seen: a central muscle that is round in transverse section, flanked on either side by wedge-shaped muscle tissue underlain by arc-like muscle profiles. The wedge-shaped muscles on either side can be identified as EDCs, and with associated arc-like EMUs. The central muscle is not easy to identify and could represent either EIL or EML. Ventrally, 6 muscles and a tendon can be seen. A small round transverse section of muscle is seen near the mid-line with on either side larger regions of muscle, and more laterally, a muscle and a tendon on the original posterior side and two muscles on the other side. The ventral arrangement is not so clearly symmetrical as the dorsal musculature. Nevertheless, we suggest that FDP, FCU and FDS are duplicated (see Fig. 1B). The shapes of muscles in a more distal section of the same limb can be seen in Fig. 6, and are consistent with the identifications given in Fig. 1B. Unlike the normal limb in which the ventral musculature is clearly asymmetrical, and the small muscles that develop anteriorly do not run the complete length



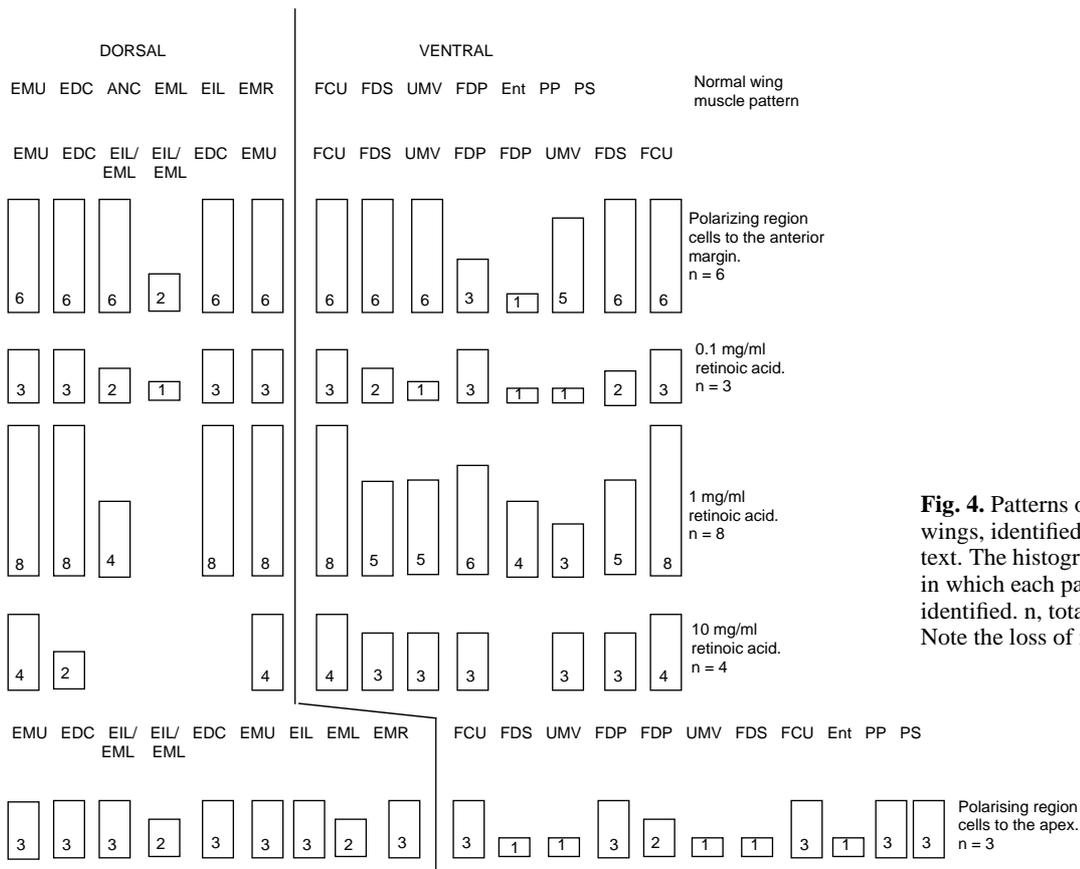
**Fig. 3.** Means for total number of muscles (open squares), number of dorsal muscles (filled circles), number of ventral muscles (filled squares) and number of digits (open circles) that develop in limbs after different doses of retinoic acid and polarizing region grafts. The mean total number of muscles and digits for normal limbs is shown by the dashed line. Each point is the mean of at least 3 cases.

of the forearm, there is a far greater muscle bulk throughout the forearm (see also Fig. 6).

As the dose of retinoic acid applied to the anterior margin of chick wing buds was increased (beads soaked in 1 mg/ml and 10 mg/ml retinoic acid), the number of muscles tended to decrease. Nevertheless, the symmetrical appearance of the muscle pattern was maintained. As shown in Figs 4 and 1D, our identification of the muscles that developed suggest that anterior muscles are lost, while posterior muscles are retained. Fewer muscles develop dorsally compared to ventrally (Fig. 1D).

When the polarising region was grafted to the apex of early wing buds, instead of to the anterior margin, the number of muscles increased (Fig. 1C). In one limb, 12 muscles were present, while in two others, 16 (Fig. 1C) and 17 muscles could be seen. Fig. 1C shows an example of the muscle pattern. Dorsally, there are 9 muscles, 6 of these lie between the two posterior skeletal elements and form a symmetrical pattern and 3 lie more anteriorly. At the very anterior of the limb, there is a large muscle lying anterior to the skeletal element, which can be unequivocally identified as EMR. The remainder of the deduced muscle identities are given in Fig. 1C.

Fig. 4 shows all the muscle pattern data obtained from manipulated limbs. Identification of EMU, EDC, FCU, FDS and FDP can be made with greater confidence than that of EIL, EML and UMV. Nevertheless, even with this limitation, it is



**Fig. 4.** Patterns of muscles in manipulated wings, identified using criteria outlined in the text. The histograms show the number of limbs in which each particular muscle could be identified. n, total number of wings examined. Note the loss of muscles especially dorsally.

clear that the order of muscles is maintained in most manipulated limbs. However, there are a few exceptions. In the dorsal muscle pattern of duplicated limbs, Anc is missing, even though either EIL or EML is present. There are also some anomalies in the ventral pattern in that FDP is duplicated even when UMV is absent.

The musculature of manipulated limbs can be compared with the skeletal pattern. The number of digits that developed is related to the dose of retinoic acid. Lower doses of retinoic acid and polarising region grafts gave most digits (Fig. 3). In contrast, the forearm of limbs with anterior grafts or retinoic acid application almost always consisted of two skeletal elements, both of which are ulnae. In one wing out of a total of 21 manipulated wings, a single forearm element developed, and this resulted from the implant of a bead soaked in 10 mg/ml retinoic acid. With polarising region grafts to the apex, 3 forearm elements developed.

**The cellular composition of muscles in the normal wing**

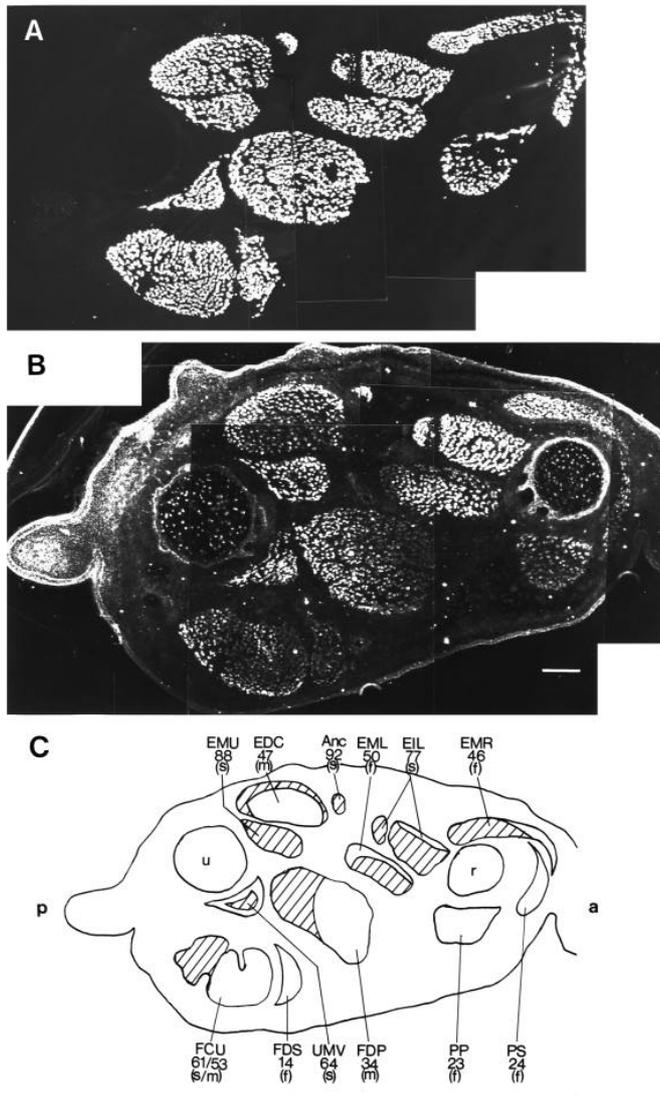
At 10 days of development, the muscles are composed mainly of primary fibres but some secondary fibres are also present. Adjacent sections were labelled with one of the monoclonal antibodies: LM5, which recognised the embryonic fast MyHC, or 98/31, which labelled a form of the slow MyHC. At this stage in development, every muscle fibre elaborates the embryonic fast MyHC, whereas only some elaborate the slow MyHC. Individual muscles of the chick wing can be classified as either fast, slow or mixed, by calculating the relative pro-

portions of the two fibre types (Fig. 5A-C). Overall, the dorsal musculature contains a larger proportion of slow fibres than the ventral musculature. The muscles in the dorsal posterior part of the limb have the highest proportion of slow fibres and those in the ventral anterior have the smallest (Fig. 5C).

Analysis of fibre type pattern in individual muscles of the normal limb show that of the dorsal anterior muscles, EMR and EML are fast, while, EIL is slow. Posteriorly, EMU, has a high proportion of slow fibres, and Anc is almost exclusively composed of slow fibres, whereas EDC is composed of a mixture of fibre types with an outer rim of slow fibres. Ventrally, anterior muscles, PS and PP, can be classified as fast muscles, while Ent is of mixed fibre type. FDP, in the centre of the limb ventrally, is also a mixed fibre type muscle, with the majority of fast fibres located in the rectangular shaped main part of the muscle, while slow fibres are located in the triangular part that points towards the ulna posteriorly. Posteriorly, UMV is a slow muscle which has a large central core of slow fibres. FDS is small and predominately composed of fast fibres. FCUs larger hook-shaped region can be classified as fast, while the smaller posterior region contains mostly slow fibres.

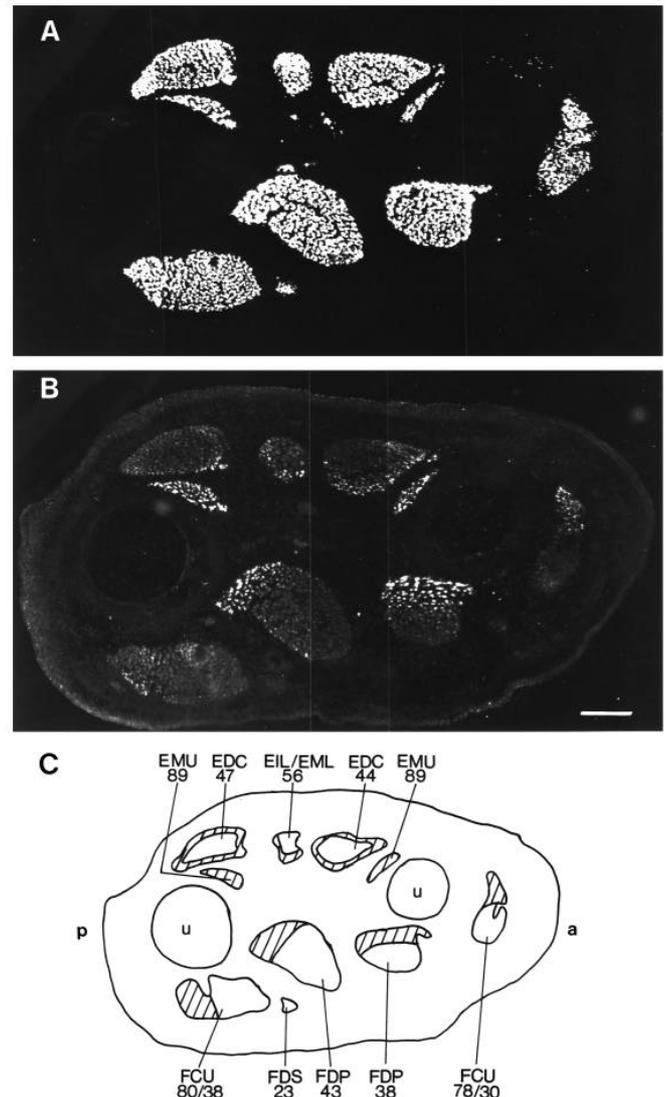
**Cellular composition of the muscles in duplicated forearms**

When a polarizing region is grafted to the anterior margin of a wing bud, the cellular composition of muscles in the anterior half of the limb mirrors the composition of muscles in the posterior half. The symmetry of muscle pattern is clearly seen in Fig. 6A-C. The muscles in this limb were identified both



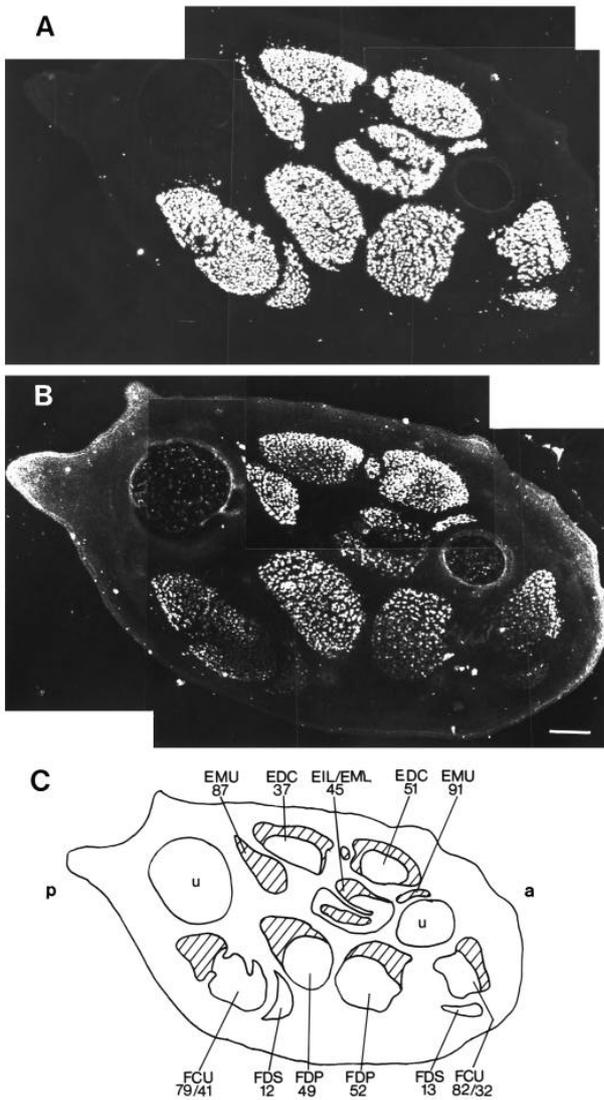
**Fig. 5.** Pattern of muscles in forearm of a normal 10 day chick wing. (A) Section immunolabelled with LM5 the antibody to the fast embryonic MyHC. (B) Adjacent section showing labelling by 98/31 the antibody to the slow MyHC. (C) Diagrammatic summary of the cellular pattern of fast and slow fibres in the muscles of the forearm. The figures below each muscle label are the percentage of slow fibres in this section. The letter in brackets is the classification of the muscle based on data from 5 limbs; (f) fast, (s) slow and (m) mixed. To classify muscles, the overall proportion of slow fibres was calculated separately for dorsal and ventral regions of the limb, and then each individual muscle was judged against its regional value. u, ulna; r, radius; a, anterior; p, posterior. Scale bar, 63  $\mu$ m.

from this section and from more proximal sections (see for example Fig. 1B). There are 5 muscles dorsally, with a central muscle which has more slow fibres ventrally. This is flanked by 2 wedge-shaped muscles in transverse section, which we suggest are EDC. Both of these EDC muscles have a peripheral rim of slow fibres. Underneath each 'EDC' is a thin elongated muscle profile (EMU) which is mostly slow. Ventrally, there are 5 muscles. There are 2 central muscles (FDP) with slow fibres located dorsally, and 2 lateral muscles (FCU) which have high slow fibre labelling in their outer



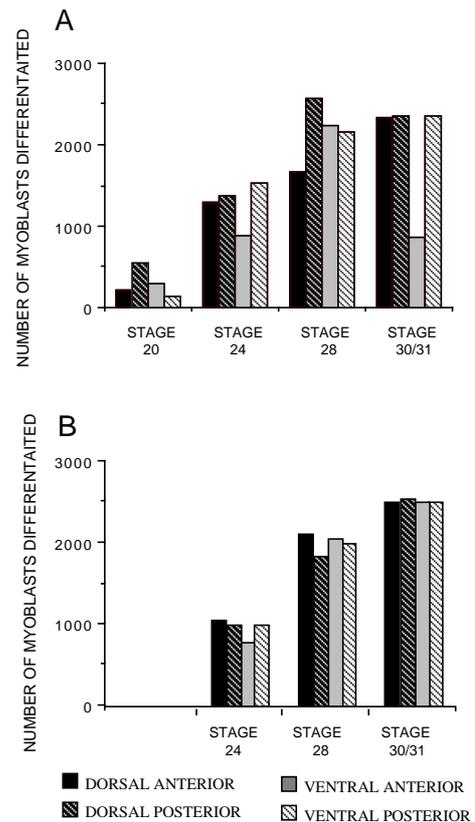
**Fig. 6.** Pattern of muscles in forearm of a wing that developed 6 days after a polarizing region graft to the anterior margin of the bud. (A) Section immunolabelled with LM5 the antibody to the fast embryonic MyHC. (B) Adjacent section showing labelling by 98/31 the antibody to the slow MyHC. The duplicated FDS is not present in this section, UMV is beginning to appear over the original FDP (Fig. 1B, shows the duplicated FDS and the central UMV). (C) Diagrammatic summary of the cellular pattern of fast and slow fibres in the muscles of the forearm. The percentage of slow fibres for each muscle in this section are shown (C). Scale bar, 75  $\mu$ m. u, ulna; a, anterior; p, posterior.

edges. A small thin muscle (FDS) with few slow fibres is located on the posterior side ventral to the central muscle in this section. A duplicate FDS is not seen anteriorly in this section, but can be seen in adjacent sections (see Fig. 1B). According to these definitions, the pattern of fast and slow fibres in duplicated muscles is clearly very similar, both with respect to proportions of different fibre types and their localization within the muscles. For example, both 'EMUs' are mainly composed of slow fibres, and the 'FCUs' have a higher proportion of slow fibres in their outer regions.



**Fig. 7.** Pattern of muscles in forearm region of a wing that developed 6 days after a bead soaked in 1 mg/ml retinoic acid was implanted at the anterior margin. (A) Section immunolabelled with LM5 the antibody to the fast embryonic MyHC. (B) Adjacent section showing labelling by 98/31 the antibody to the slow MyHC. (C) Diagrammatic summary of the cellular pattern of fast and slow fibres in the muscles of the forearm. The figures show the percentage of slow fibres for each muscle in this section. (Fig. 5 shows the contralateral left limb). Scale bar, 75  $\mu$ m. u, ulna; a, anterior; p, posterior.

Symmetrical patterns of duplicated muscles with characteristic spatial ordering of fast and slow fibres also develop following implantation of beads soaked in retinoic acid. Fig. 7A-C shows an example of a limb following implantation of a bead soaked in 1 mg/ml retinoic acid (Fig. 5A-C is the normal contralateral limb). The muscle pattern on the dorsal side appears to be duplicated and the arc-shaped muscle lying immediately next to the skeletal element on both sides appears to be composed of mainly slow fibres, and we identified these as duplicated EMU muscles. The muscles that appear in transverse section on either side in the dorsal midline have a rim of



**Fig. 8.** Mean number of myogenic cells that differentiate in micromass cultures from the four regions of (A) normal limbs, stages 20 to 30-31; (B), limbs treated with 1 mg/ml retinoic acid, stages 24, 28 and 30-31. The data were collected from 2 cultures at stage 20, and 4 cultures for all other stages, myogenic cells were labelled with LM5 (fast embryonic MyHC) and 98/31 (slow MyHC).

slow fibres. Ventrally, the pattern of muscles is even more clearly altered from the normal limb, and a very symmetrical arrangement of 6 muscles is found. On each side there is a large muscle at the edge of the limb which has an outer region containing mostly slow fibres. Immediately adjacent to these muscles on either side, is a small muscle that has few slow fibres. We interpret these as representing duplicate muscles of FCU and FDS respectively.

Quantitative analysis of the cellular composition of muscles of limbs treated by implantation of a bead soaked in 1 mg/ml retinoic acid confirms that the proportions of fast and slow fibres are the same in both original and duplicate EMUs, and the posterior regions of FCU muscles (Table 1). The numbers represent the proportions of slow fibres in named muscles for several different limbs. The data for each pair of muscles in each limb were statistically analysed by applying the Wilcoxon test which showed that differences between proportions of fast and slow fibres were not significant. The proportion of fast and slow fibres in individual duplicated muscles was compared with the same muscle in the contralateral control limb by the Friedmann's test. These statistical analyses show that there is no significant difference in the population of slow fibres in muscles of each contralateral limb and both original and duplicated muscles of the corresponding manipulated limb.

**Micromass cultures of normal and retinoic acid-treated limbs**

To map the distribution of potentially myogenic cells in developing limbs, micromass cultures of cells were prepared from four presumptive forearm regions of stages 24, 28 and 30-31 limbs; dorsal anterior and dorsal posterior regions, and ventral anterior and posterior regions. The cells were cultured for 2.5 days and then myoblasts expressing either fast or slow MyHC in parallel cultures were recognized by immunolabelling. The total numbers of myoblasts that differentiated and the percentage of these that were labelled by the slow MyHC antibody are shown in Fig. 8 and Table 2.

At all four stages, there is a significant population of potentially myogenic cells within the limb bud, but the number of myoblasts that differentiate in cultures steadily increases between stages 20 and 28. At all four stages, a similar total number of myoblasts differentiate within the four regions, except at stage-24 and 30-31 when there are fewer myoblasts in the ventral anterior region (the region in the normal limb, in which fewest muscles develop). The smallest number of slow myoblasts differentiate in ventral posterior cultures; the highest number in dorsal anterior cultures (Table 2). However, in the limb, the dorsal posterior region has the highest number of slow fibres and the ventral anterior, the lowest. Nevertheless, cultures from the dorsal regions of the wing contain overall a higher proportion of slow fibres than ventral cultures, which mirrors fibre type distribution in the wing musculature.

To investigate the response to retinoid treatment of the population of potentially myogenic cells in early limbs, numbers of potentially myogenic cells and proportions of fast and slow myoblasts were determined 24, 48 and 90 hours after treatment of stage-20 limbs. The limbs were divided into the same four regions used for the normal limbs. In stage-24 and stage-28 (24 and 48 hours) cultures made from the retinoic acid-treated limbs, fewer myogenic cells differentiate than in micromasses from untreated limbs of the same stage (Fig. 8). However, as in normal limbs, there is an increase between stages 24 and 28 in the total number of myoblasts that differentiate. At stage 30-31, the number of myoblasts that differentiate from the ventral anterior region is similar to that from the other three regions, whereas in the same region from normal limbs there is a very clear drop in its myogenic population (Fig. 8). This maintenance of muscle cell number may be associated with the development of duplicate muscles in this region of the manipulated limb. With respect to fibre types, there are no differences in proportions of slow myogenic cells in cultures from retinoid-treated limbs prior to stage 30/31, compared with normal limbs. The populations of the slow myogenic cells within the two anterior region cultures at stage 30/31 are reduced and may reflect the beginning of an alteration in the cellular patterning (Table 2).

**Splitting of muscle masses in normal and duplicated limbs**

The musculature of the limb arises from sequential splitting of two muscle masses, one dorsal and the other ventral as shown in Fig. 9 (see also Shellswell and Wolpert, 1977). In manipulated limbs that will produce a duplicated forelimb musculature, the splitting pattern of the muscle masses is altered. Ventrally, at stage 28, at the end of the first round of splitting, there are three muscle masses in the distal region of the forearm of duplicated limbs whereas normally there are only two (compare Fig. 10E with Fig. 10A). The additional muscle mass under the anterior cartilage element could give rise to duplicate FCU and FDS muscles. The large muscle mass in the centre of the limb could split into either FDP or UMV muscles, while the most posterior muscle mass could provide the normal FDS and FCU muscles on the posterior margin of the limb. Dorsally, there seems to be a delay in the start of splitting. Even by stage 30 (Fig. 10G) there are still no clear divisions in the dorsal muscle mass, whereas by this stage in the contralateral limb (Fig. 10C) individual muscles are forming.

In the normal limb, there are more slow fibres in the posterior region of the dorsal muscle mass (Fig. 10B). Ventrally, there are more slow fibres in the posterior tip of the large central muscle mass (Fig. 10B). These areas will eventually produce muscles with high proportions of slow fibres,

**Table 1. Wilcoxon (A and C), and Friedmann's (B and D) statistical analysis for EMU, and the posterior region of FCU**

A		C	
Original FCU	75 78 81 79 88 88	Original EMU	69 79 87 91 80 80
Duplicated FCU	73 81 76 82 62 78	Duplicate EMU	73 77 90 87 85 75
Difference (original - duplicate)	+2 -3 +5 -3 +26 +10	Difference (original - duplicate)	-4 +2 -3 +4 -5 -5
Rank (R)	1 2.5 4 2.5 6 5	Rank (R)	3.5 1 2 3.5 5.5 5.5
R+	1 4 6 5	R+	1 3.5
R-	2.5 2.5	R-	3.5 2 5.5 5.5
R+ (16) + R- (5) = 21 n=6		R+ (4.5) + R- (16.5) = 21 n=6	
T=5 not significantly different		T=4.5 not significantly different	
B		D	
Control FCU	73 61 80 79	Control EMU	92 88 78 80
Original FCU	81 79 88 88	Original EMU	87 91 80 72
Duplicate FCU	76 82 62 78	Duplicate EMU	90 87 80 75
Rank	2 1 4 3 2 1 3.5 3.5 2 4 1 3	Rank	4 3 1 2 3 4 1.5 1.5 4 3 2 1
Total	6 6 8.5 9.5 = 30	Total	11 10 4.5 4.5 = 30
S = 36 + 36 + 72.25 + 90.25 - $\frac{30^2}{4}$		S = 121 + 100 + 20.25 + 20.25 - $\frac{30^2}{4}$	
= 234.5 - 225 = 9.5		= 261.5 - 225 = 36.5	
n = 4 m = 3 S at the 5% level is 37		n = 4 m = 3 S at the 5% level is 37	
The difference is not significantly different.		The difference is not significantly different.	
The Wilcoxon compares medians from two populations, in this case original and duplicated muscles, in limbs treated with 1 mg/ml retinoic acid. The Friedmann's compares medians from more than two populations which are the same except for their location, in this case, muscles in contralateral left limbs are compared with muscles in retinoid-treated limbs, both original and duplicates.			

for example EMU and Anc in the dorsal posterior region, and UMV and the slow tip of FDP ventrally. In the duplicated limb, the pattern of cellular differentiation appears to be the same as in the normal limb at stage 28 (compare Fig. 10F with 10B). Even by stage 30 there is little change in the fibre type pattern in the manipulated limbs. For example, ventrally, the number and distribution of slow fibres in the muscle masses is the same as that seen in the untreated limb even though the pattern of splitting has been altered (compare Fig. 10D with 10H).

**DISCUSSION**

The wing bud can be manipulated by either grafting a polarizing region or local application of retinoic acid to the anterior margin. In the forearm of manipulated wings, 2 ulnae develop and there is a mirror image pattern of posterior muscles. When the wing is manipulated by application of higher doses of retinoic acid, fewer muscles form but the muscle pattern remains symmetrical and anterior muscles are lost. There is an approximate correspondence between the number of forearm elements and the number of muscles that develop. Each muscle of the forearm of the chick wing has a characteristic pattern of fast and slow fibres. In duplicated limbs, the new ‘posterior’ muscles resemble their normal counterparts not only with respect to their arrangement and shape but also with respect to their cellular pattern of fast and slow fibres. In duplicated limbs, the alteration in splitting of muscle masses that generates tissue pattern is the first detectable change in the development of the musculature. The population of fast and

**Table 2. Percentage of slow myogenic cells that differentiated in micromass cultures after 2.5 days**

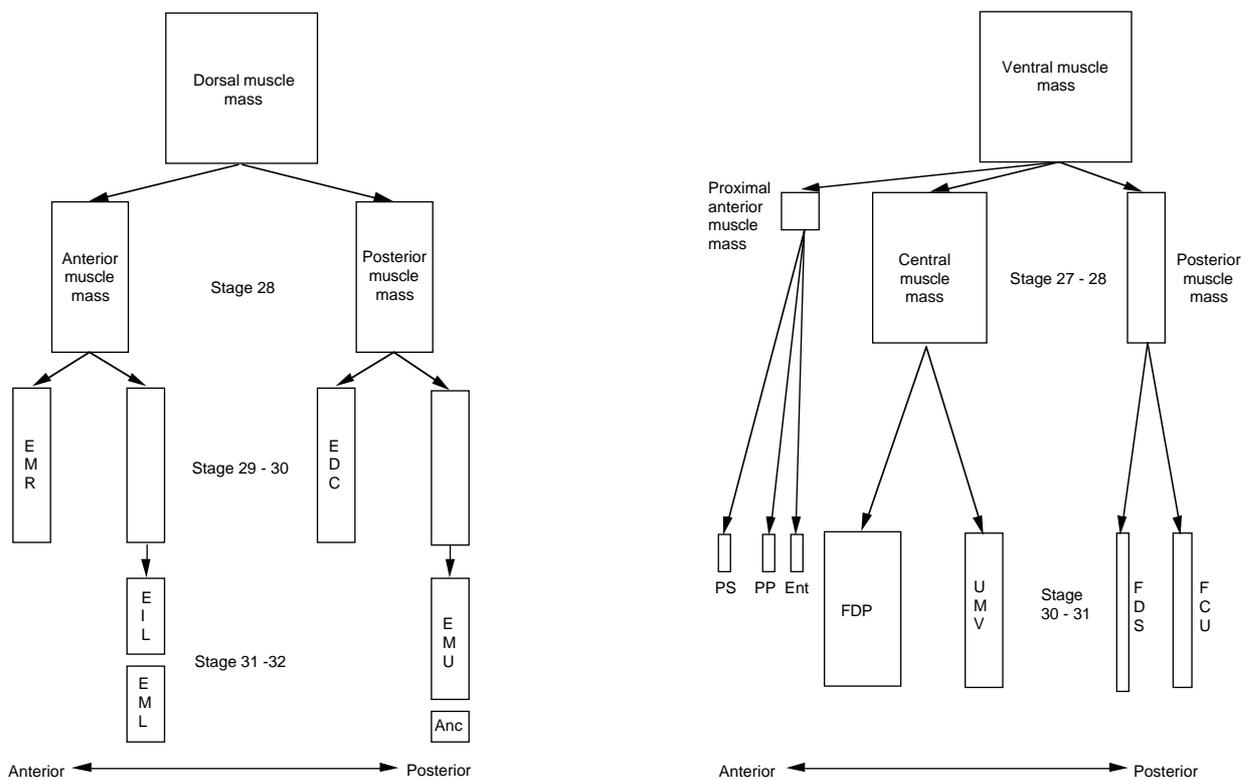
Stage of limb buds	Dorsal anterior	Dorsal posterior	Ventral anterior	Ventral posterior
<b>A</b>				
20	71	47	46	25
24	57	29	36	18
28	58	33	39	20
30/31	27	18	30	16
<b>B</b>				
24	56	34	29	16
28	51	33	38	22
30/31	19	13	17	16

Normal untreated limb cultures are shown in A; cultures from limbs treated with 1 mg/ml retinoic acid soaked beads at stage 20 are shown in B. The only variation seen in the proportions of slow myogenic cells in the anterior cultures is 90 hours after retinoid application.

slow potentially myogenic cells that generates the cellular pattern of the muscles does not change until later.

**Muscle patterning at the tissue level**

Grafts of polarizing region cells or application of low doses of retinoic acid to the anterior margin of early wing buds lead to mirror image skeletal patterns, which are particularly clearly seen in the digits (Tickle et al., 1975; Tickle et al., 1982). In the forearm of these manipulated limbs, two elements develop as normal but the anterior element is an ulna rather than a radius. Here, we show that the pattern of muscles in the forearm also has a mirror-image symmetry (see also Shellswell



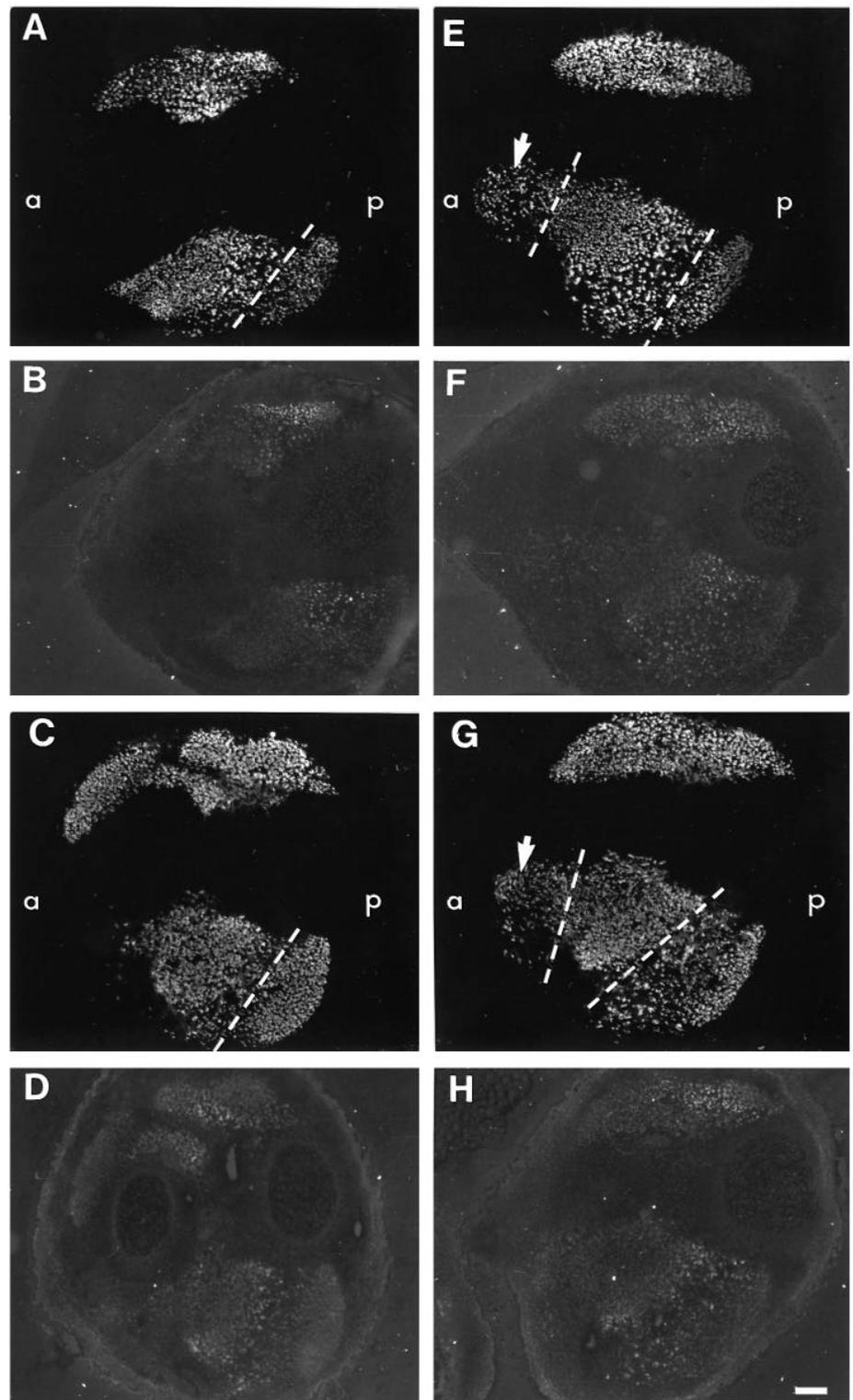
**Fig. 9.** Schematic diagram of the splitting process for both dorsal and ventral muscle masses.

and Wolpert, 1977). There is little or no increase in the number of muscles and, from the symmetry of the muscles, it can be deduced that a duplicate set of 'posterior' muscles has now developed at the anterior of the limb.

Similar rules appear to govern both skeletal and muscle duplication. For example, anterior structures can be respecified to form posterior structures but posterior cannot form anterior. In addition, just as the anterior digit, digit 2, is lost when increasing doses of retinoic acid are applied to limb buds so too are 'anterior' muscles. However it is not clear why dorsal muscles are lost more readily than ventral muscles. For the digit pattern, the order of the digits is always maintained so that for example, a digit 2 develops next to a digit 3 or another digit 2, but never next to a digit 4. This rule does not appear to be so inviolate for the musculature. In several cases, it appeared that a duplicate FDP developed but no UMV. Dorsally, Anc, is almost always absent, and a central muscle, which appears to be either EML or EIL, is formed. Such inconsistencies of muscle 'ordering' in duplicated limbs appear to be related to the size of the muscles. The smallest muscle formed by splitting of a muscle mass tends to be lost, for example, FDS ventrally, whereas the larger muscle is present, for example, FCU. If the smaller muscle is the more posterior of the two muscles generated by the split, then there is a gap in muscle pattern. It is interesting that loss of these muscles is symmetrical, and that the muscle is also missing on the non-respecified side.

### Cellular patterning of muscles

Each muscle has a characteristic cellular pattern of fast and slow fibres, which is laid down early in development in the primary fibres. In manipulated limbs, in which tissue pattern is duplicated, muscles at the anterior of the limb which appear to be duplicates of posterior muscles have the same patterns of fast and slow fibres as their posterior counterparts. Both the proportions of fast and slow fibres and the distribution of the two fibre types within the pairs of duplicated muscles are similar. In addition, the proportion of fibre types in any named muscle and its duplicate is the same as that in the muscle in the contralateral unoperated



**Fig. 10.** Normal (A-D), and 1 mg/ml retinoid-treated limbs (E-H). A,E (stage 28), C,G (stage 30-31) labelled with LM5, B,F (stage 28), D,H (stage 30-31) labelled with 98/31. At stage 28, in the normal limb (A,B) 2 muscle masses can be seen ventrally; dorsally the first division has just begun. At stage 30-31, in normal limbs individual muscles can be seen dorsally (C,D). At stage 28 in the duplicated limb there is no sign of any splitting dorsally (E,F), 3 muscle masses can be seen ventrally (dashed lines indicate divisions of the ventral mass), with a new mass (arrow) under the anterior cartilage element. At stage 30-31 there is still no apparent splitting dorsally (G,H). The distribution of slow fibres in retinoid-treated and normal limbs appears to be the same at both stages. Scale bar, 10  $\mu$ m, a, anterior; p, posterior.

wing. Therefore the duplicate muscle appears to faithfully reproduce the detailed cellular pattern.

### Muscle number

The number of muscles in the forearm bears some relationship to the number of skeletal elements. When three forearm bones are present, the number of muscles is increased, whereas when only one bone is present, the number is decreased. In the normal limb, 7 named muscles are associated with the ulna and 6 named muscles with the radius. If skeletal pattern directly controls muscle pattern, we would expect 14 muscles in limbs with 2 ulna and 20 muscles in limbs with 2 ulnae and one radius. However, the limbs had consistently fewer muscles than predicted. Therefore skeletal pattern does not appear to dictate directly muscle number (see also Shellswell and Wolpert, 1977).

### Myogenic cells in culture and muscle construction

When cells from early limb buds are cultured at high density, myoblasts differentiate and both fast and slow MyHC's can be expressed (Miller et al., 1985; Miller and Stockdale, 1986). We used this technique to map the distribution of the potentially myogenic pool in the developing limb bud (see also Archer et al., 1992). A greater number of slow myoblasts differentiated in cultures from the dorsal regions of the limb, reflecting the composition of the dorsal muscles. However, the proportion of myogenic cells in anterior and posterior cultures that express the slow MyHC appears to be the opposite of the proportion of slow fibres in the muscles that develop there. Therefore, the construction of the musculature of the limb does not appear to involve simply gathering up locally available fast and slow myoblasts by connective tissues to give correct overall fibre type proportions. This contrasts with the intermandibularis muscle of the lower jaw, where proportions of fast and slow myoblasts in cultures matches fibre type distribution in the muscle (Robson, 1993).

In the developing limb, differentiation of myoblasts occurs during the splitting process. Analysis of fast and slow fibre distribution during splitting shows that appropriate distributions of fast and slow fibres begin to appear within the muscle masses. It is possible that cells sort within these masses. However, the behaviour of marked myoblasts within the limb bud suggest that there is little or no movement in the appropriate directions (Lee and Ede, 1990). Another possibility is that myogenic cells change their fibre type expression.

When the time scale for the various events in muscle patterning in manipulated limbs are analysed, it can be seen that tissue pattern is respecified earlier than cellular pattern. We found that tissue patterning of muscle is detectably changed by at least 48 hours after the manipulation, as seen by the altered splitting pattern. However, cellular patterning, which gives the arrangement of fibre types within the new 'posterior' muscles, does not appear to be altered until at least 90 hours after the manipulation. At this time, splitting is complete, and secondary fibres have begun to differentiate (Fredette and Landmesser, 1991). Therefore, the patterning of fibre types in duplicated muscles may reflect the arrangement of secondary myoblasts. This suggests that secondary fibres may recognise the new identity of muscles without reference to the primary fibre pattern. These patterns of secondary myoblasts could be established by interactions with connective tissue sheaths of muscles

or by the pattern of innervation, leading to selective cell death and/or proliferation of relevant myoblast types.

This work was supported by the MRC. T. K. was a medical student engaged on a project as part of an intercalated BSc degree supported by the MRC. The authors thank Dr Geoff Shellswell for advice on muscle patterns in retinoid-treated limbs and Dr Simon Hughes and Prof. L Wolpert for reading the manuscript.

## REFERENCES

- Archer, C. W., Langille, R. M., Teran, M. A. F. and Solorsh, M. (1992). Myogenic potential of chick limb bud mesenchyme in micromass culture. *Anat. Embryol.* **185**, 299-306.
- Brickell, P. and Tickle, C. (1989). Morphogens in chick limb development. *Bioessays* **11**, 145-149.
- Butler, J., Cosmos, E. and Cauwenbergs, P. C. (1988). Positional signals: evidence for a possible role in muscle fibre-type patterning of the embryonic avian limb. *Development* **102**, 763-772.
- Chevallier, A. and Kieny, M. (1982). On the role of the connective tissue in the patterning of the chick limb musculature. *Wilhelm Roux's Archiv. Dev. Biol.* **191**, 277-280.
- Crow, M. T. and Stockdale, F. E. (1986). Myosin expression and specialization among the earliest muscle fibres of the developing avian limb. *Dev. Biol.* **113**, 238-254.
- Dhoot, G. K. (1989). Evidence for the presence of a distinct embryonic isoform of the myosin heavy chain in chicken skeletal muscle. *Differentiation*. **40**, 176-183.
- Dhoot, G. K., Dransfield, I., Grand, R. J. A. and Perry, S. V. (1988). Distribution of isoforms of the myofibrillar proteins in myoid cells of the thymus. *J. Muscle Res. Cell Motil.* **7**, 351-360.
- Fredette, B. J. and Landmesser, L. T. (1991). Relationship of primary and secondary myogenesis to fibre type development in embryonic chick muscle. *Dev. Biol.* **143**, 1-18.
- Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49-92.
- Hilfer, S. R., Searls, R. L. and Fonte, V. G. (1973). An ultrastructural study of early myogenesis in the chick wing bud. *Dev. Biol.* **30**, 374-391.
- Jacob, M., Christ, B. and Jacob, H. J. (1978). On the migration of myogenic stem cells into the prospective wing region of chick embryos. *Anat. Embryol.* **153**, 179-193.
- Kenny-Mobbs, T. (1985). Myogenic differentiation in early chick wing mesenchyme in the absence of brachial somites. *J. Embryol. exp. Morph.* **90**, 415-436.
- Lee, K. K. H. and Ede, D. A. (1990). An autoradiography study of myogenic cell movement in avian limb buds following heterospecific and homospecific transplantation. *Anat. Embryol.* **182**, 547-551.
- Miller, J. B., Crow, M. T. and Stockdale, F. E. (1985). Slow and fast myosin heavy chain content defines three types of myotubes in early muscle cell cultures. *J. Cell. Biol.* **101**, 1643-1650.
- Miller, J. B. and Stockdale, F. E. (1986). Developmental regulation of the multiple myogenic cell lineages of the avian embryo. *J. Cell. Biol.* **103**, 2197-2208.
- Ordahl, C. P. and Le Douarin, N. M. (1992). Two myogenic lineages within the developing somite. *Development* **114**, 339-353.
- Robson, L. G. (1993). The cellular patterning of fast and slow fibres in the intermandibularis muscle of the chick embryo. *Development* **113**, 329-339.
- Saunders, J. W. and Gasseling, M. T. (1968). Ectoderm-mesenchymal interactions in the origin of wing symmetry. In *Epithelial-Mesenchymal Interactions* (ed. R. Fleischmajer and R. E. Billingham), Baltimore, Maryland: Williams and Wilkins.
- Solorsh, M., Drake, C. and Meier, S. (1987). The migration of myogenic cells from the somites at the wing level in avian embryos. *Dev. Biol.* **121**, 389-396.
- Schramm, C. and Solorsh, M. (1990). The formation of premuscle masses during chick wing bud development. *Anat. Embryol.* **182**, 235-247.
- Shellswell, G. B. (1977). *Studies on the Development of the Pattern of Muscles and Tendons in the Chick Wing*. Ph.D. thesis, University of London.
- Shellswell, G. B. and Wolpert, L. (1977). The pattern of muscle and tendon development in the chick wing. In: *Vertebrate Limb and Somite Morphogenesis*. (ed. D. A. Ede, J. R. Hinchliffe and M. Balls) Cambridge: Cambridge University Press.

- Sullivan, G. E.** (1962). Anatomy and embryology of the wing musculature of the domestic fowl (*Gallus*). *Aust. J. Zool.* **72**, 458-518.
- Sweeney, L. J., Kennedy, J. M., Zak, R., Kokjohn, K. and Kelley, S. W.** (1989). Evidence for expression of a common myosin heavy chain phenotype during initial stages of avian embryogenesis. *Dev. Biol.* **133**, 361-374.
- Tickle, C.** (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. *Nature* **289**, 295-298.
- Tickle, C.** (1991). Retinoic acid and chick limb bud development. *Development Supplement*, 113-121.
- Tickle, C., Summerbell, D. and Wolpert, L.** (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* **254**, 199-202.
- Tickle, C., Alberts, B., Wolpert, L. and Lee, J.** (1982). Local application of retinoic acid to the limb bud mimics the action of the polarizing region. *Nature* **296**, 564-566.
- Tickle, C., Lee, J. and Eichele, G.** (1985). A quantitative analysis of the effects of all-*trans*-retinoic acid on the pattern of chick wing development. *Dev. Biol.* **109**, 92-95.

(Accepted 20 January 1994)