

Cellular patterning of fast and slow fibres in the intermandibularis muscle of chick embryos

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SUMMARY

The way in which the pattern of cell types arises during development of individual muscles was explored. The pattern of cellular differentiation resulting from the synthesis of particular fast and slow myosin heavy chains (MyHC) was investigated in the intermandibularis muscle in the lower jaw of chick embryos. The intermandibularis muscle has a proximodistal pattern of fibre type distribution. The distal region of the muscle contains a ratio of 1.5:1 fast to slow muscle fibres, which increases to >2.5:1 in the proximal region. The intermandibularis muscle is assembled in a proximodistal sequence, with both fast and slow muscle cells differentiating within the earliest muscle and then establishing the specific pattern of cell types. This pattern is not

dependent on a specific innervation source, as normal lower jaw muscles develop and the intermandibularis has the same graded cellular pattern when the mandibular primordium is grafted to the limb bud stump. Micromass cultures were used to explore the pool of potentially myogenic cells that are available to construct the muscles. Even before the muscle differentiates *in vivo*, both fast and slow cells are present in the primordia. These potentially myogenic cells are already distributed within the primordium in a proximodistal fashion that mimics the cellular pattern found in the muscle that develops.

Key words: myogenesis, fibre types, muscle development

INTRODUCTION

The development of the muscle is a complex process and involves patterning of the muscles and patterning of the cells within the individual muscles. At the tissue level, patterning involves the formation of a number of discrete muscles at appropriate locations in the embryo. The myogenic cells that form the skeletal muscles are derived from the paraxial mesoderm in the head (Noden, 1983; Couly et al., 1992) and from the somitic mesoderm in the trunk (Ordahl and Le Douarin, 1992). Myogenic cells migrate into the regions of the head and limbs where muscle will eventually develop (Ordahl and Le Douarin, 1992). There is good evidence that the pattern of discrete muscles is determined by the connective tissue. When regions of paraxial mesoderm or particular somites are transplanted to a new location early in development, the myogenic cells migrate and become arranged into the pattern of muscles appropriate to their new location. Noden (1986), for example, showed that, when cervical somites were grafted to the head region, the muscles that formed appeared to be normal lower jaw muscles, even though the myogenic cells were derived from a foreign origin. The connective tissue seems to direct the assembly of myogenic cells into the appropriate spatially ordered arrangement of muscles.

The second patterning process in early muscle development involves the spatial ordering of differentiated cells within individual muscles, to give for example, a precise arrangement of discrete fibre types. Cultures of early limb buds and of the facial primordia show that potentially myogenic cells are present long before muscle differentiates *in vivo*, and that both the fast and the slow MyHCs are expressed in these early stages (Miller et al., 1985; Ralphs et al., 1989). There is some evidence that local conditions determine this pattern of cellular differentiation within the individual muscles. For example, Butler et al. (1988) transplanted unsegmented thoracic somitic mesoderm to sites opposite the presumptive limb region. The resulting musculature that developed within the limb buds had essentially the normal pattern of fast and slow fibres. Although innervation plays an important role in the later stages of muscle development, specifying the final configuration of fast and slow fibres, its role in early muscle development would seem to be limited (Crow and Stockdale, 1986).

This study focuses on cellular patterning in the development of the intermandibularis muscle of the lower jaw of chick embryos. The pattern of cellular differentiation in terms of fast and slow fibre types has been mapped in the intermandibularis, with specific skeletal muscle myosin heavy chain (MyHC) antibodies. The way in which this cel-

ular pattern arises within the muscle has been traced during development. The distribution of potentially fast and slow myogenic cells within early primordia has been investigated in cell culture. In addition, the effect of changing the source of innervation on muscle fibre type distributions has been assessed.

MATERIALS AND METHODS

Immunolabelling of the muscles of the lower jaw

The mandibular primordia from stages 27, 28, 29/30 and 36 chick embryos (Hamburger and Hamilton, 1951) were dissected into 5% sucrose in 0.1 M phosphate buffer (pH 7.4). The primordia were taken through a graded series of sucrose solutions, made from differing proportions of 5 and 20% sucrose solutions in 0.1 M phosphate buffer (2:1, 1:1 and 1:2), the tissue was placed in each new sucrose solution for half an hour. Finally the primordia were placed in a 20% sucrose solution in 0.1 M phosphate buffer, for several hours until they had sunk to the bottom of the dish. When the primordia had sunk, they were placed in a 2:1 mixture of 20% sucrose solution and OCT compound (BDH) and left to infiltrate for half an hour, after which they were transferred to small moulds containing fresh 2:1 20% sucrose:OCT compound and frozen in liquid nitrogen. Cryosections were cut at 7 μm on a Reichert "Frigocut" cryostat and picked up on gelatin-subbed slides.

The sections were stored at -20°C for no longer than 48 hours before fixation in cold 70% ethanol for 10 minutes, then labelled with one of the four monoclonal antibodies. All the muscle-specific monoclonal antibodies were the kind gift of Dr G. K. Dhoot, the method for preparation and specificities of all the monoclonal antibodies used have been described elsewhere (Dhoot, 1986, 1988; Dhoot et al., 1986; Kilby and Dhoot, 1988). The slow MyHC antibody 98/31 has not been reported previously but appears to label the same population of slow fibres as 98/12 (personal communication with Dr Dhoot) These were (working strength in brackets) 83B6, recognizing all striated muscle MyHCs (ascites 1:100); LM5, which labels the fast skeletal MyHCs from all developmental stages (ascites 1:500); 96J, an antibody that recognizes the slow embryonic MyHC and SM1 (one of the slow skeletal MyHCs; ascites 1:200) and 98/31 which only picks out the SM1 MyHC (ascites 1:60). A neurofilament antibody to the $68 \times 10^3 M_r$ protein was also used to detect the presence of nerves within the lower jaw (Amersham 1:5). All the antibodies were diluted in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, 0.05% sodium azide and 0.05% Tween 20. The procedure was the same for each of the antibodies. After fixation the sections were rehydrated with PBS and incubated with one of the primary antibodies for 1 hour. Controls were incubated with PBS or nonimmune serum. The primary antibody was washed off with PBS and a second antibody was applied to the sections for 45 minutes, this was a FITC-conjugated rabbit anti-mouse IgG (Dakopatts 1:50). After washing with PBS, the slides were mounted in 9:1 glycerol/PBS containing 2.5% 1,4-diazobicyclo-(2,2,2)-octane at pH 8.5 as a fluorescence preservative. The slides were viewed under a Zeiss photomicroscope III, with an epifluorescence attachment.

Grafting of mandibular primordia tips

The mandibular primordia from stages 20 and 24 were dissected into culture medium (Minimum Essential Medium + 10% fetal calf serum (FCS) + 4 mM L-glutamine + 200 units/ml penicillin, 200 $\mu\text{g}/\text{ml}$ streptomycin and 0.5 μg fungizone (GIBCO Biotect antibiotic/antimycotic)). The host embryo's right limb bud was

removed with sharp tungsten needles, and the fused distal anterior tips of a mandibular primordia was pinned onto the limb bud stump with small platinum pins (Fig. 1 shows the size of the grafted tips). The grafts were examined after 7-8 hours later and, if the graft had healed onto the stump, then the pins were carefully removed. The grafted embryos were then left for a total of 6 days before the embryo was fixed in formol saline, stained with alcian blue and cleared in methyl salicylate. The cleared whole-mount embryos were examined between crossed Polaroid filters; muscle being birefringent, appears bright against a dark background. The grafted mandibular outgrowths were measured using an eye piece graticule, and then photographed under normal light. A few grafts were then embedded in wax and sectioned at 7 μm , stained with Mallory's trichrome method to visualise the muscle within the grafts. Some unfixed grafted primordia were cryosectioned, having first passed through the same graded sucrose solutions as described earlier, and then labelled for fast and slow muscle fibres with the skeletal muscle antibodies.

To assess the origin of the resulting outgrowth, quail/chick grafts were carried out. These grafts were fixed in Carnoy's for a maximum of 12 hours, and embedded in wax for sectioning. These were also sectioned at 7 μm and then stained with haematoxylin (pretreatment with 6 N HCl at 60°C) and counterstained with eosin for visualization of the quail nucleolar marker (Hutson and Donahoe, 1984).

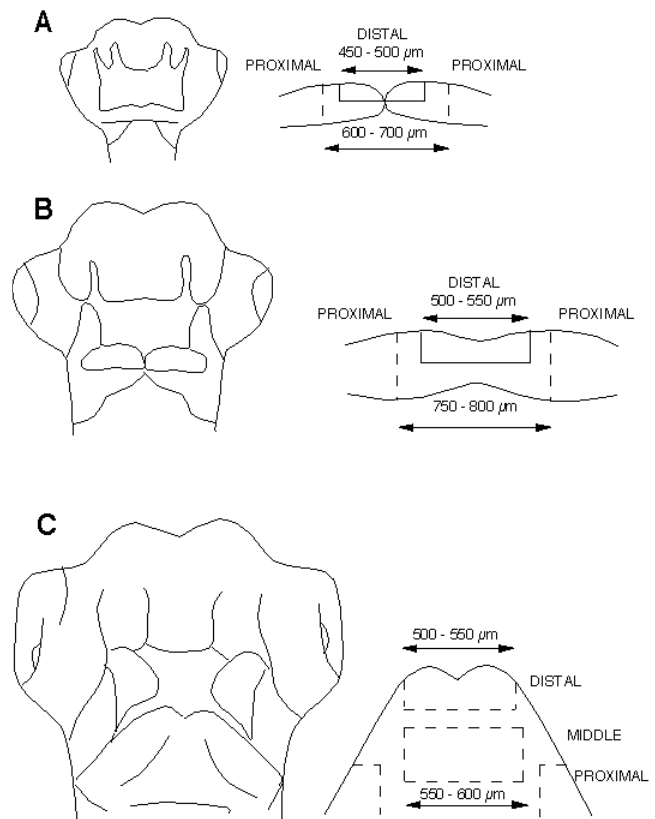


Fig. 1. Diagrammatic representation of the developmental stages of the facial primordia (not to scale); (A) stage 20, (B) stage 24 and (C) stage 28. The mandibular primordia are shown in greater detail for each stage and show the locations of the pieces used for grafting to the limb bud stump (solid lines), while the regions cut for the micromass cultures are indicated (dashed lines).

Cell culture

Micromass cultures were prepared from the mandibular primordia of stages 20, 24 and 28 chick embryos. The primordia were dissected out in calcium- and magnesium-free saline (CMF) + 10% FCS, and then dissected into proximal and distal regions as shown in Fig 1. The ectoderm was removed from the tissue by treatment with 2 % trypsin at 4°C for 45 minutes. The mesenchyme was then placed in cold CMF + 10% FCS, suspensions of each mandibular region were made by pipetting the tissue fragments vigorously. The cells were spun down at 3000 g for 3 minutes, then resuspended in 1 ml of CMF + 10% FCS. The number of cells per unit volume was calculated before the cells were spun down again, and then resuspended in Defined medium (60:40 Hams F12: DMEM + 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone (GIBCO Biocult antibiotic/antimycotic) + 5 µg/ml insulin, 5 µg/ml transferrin, 100 nM hydrocortisone and 50 µg/ml ascorbic acid). The final volume was adjusted to give 2×10^5 cells per 10 µl. The cell suspensions were then pipetted in 10 µl drops into 15 mm diameter 4-well tissue-culture grade dishes (one drop per well) and incubated at 38°C with 5% CO₂. After 1 hour, the cultures were flooded with 200 µl of fresh medium and returned to the incubator. The culture medium was replaced every 24 hours and all cultures were grown for 2.5 days.

Immunolabelling of myogenic cells in culture

Myogenic cells were identified in cultures using the same panel of muscle-specific monoclonal antibodies as above. Cultures were fixed in cold 70% ethanol for 5 minutes, then rehydrated with PBS three times over 10 minutes. The primary antibodies were incubated with the cultures for 1 hour. The cultures were then washed with PBS three times over 10 minutes, and the second antibody, goat anti-mouse 5 nM colloidal gold IgG (Biocell 1:50) was applied and incubated for 45 minutes. The cultures were then washed with distilled water and the cultures were enhanced with silver (Sigma silver enhancing kit) in the semidark until the muscle cells were darkly stained. The cultures were then washed with distilled water and stored under glycerol. Muscle cells could be counted under a Zeiss Axiovert 405M inverted microscope.

RESULTS

Development of muscles in the lower jaw

The intermandibularis is a major muscle of the lower jaw, and is well developed by 10 days of development (stage 36; Fig. 2). It spans the two Meckel's cartilages and runs from the tip to the base of the lower jaw. It is attached to the upper surface of Meckel's cartilage on each side, and there is a central raphe down the midline. The fibres of the intermandibularis muscle are orientated so that they run across the lower jaw. The pattern of cellular differentiation in this muscle was examined at 10 days of development using antibodies to all skeletal myosins (83B6), the fast (LM5) and slow (98/31) MyHCs. The antibody 83B6 and LM5 labelled all the fibres, whereas 98/31 labelled about 30% of the fibres (Table 1). Therefore the muscle is composed of a mixture of myogenic cells, all of which express the fast (fast fibres) and some the fast and slow MyHCs (slow fibres). At the distal tip of the intermandibularis, a ratio of 1.4:1 fast to slow fibres was found. This ratio gradually increased more proximally, so that at the base of the

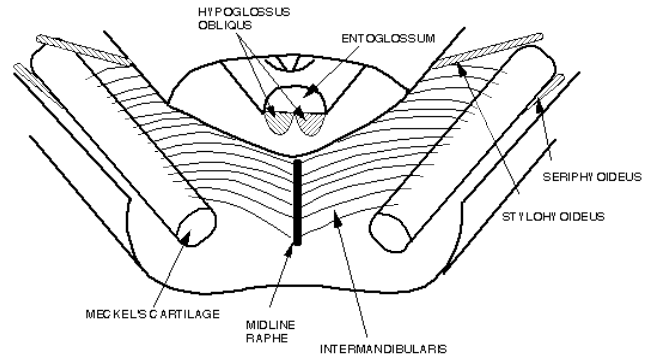


Fig. 2. A schematic representation of the lower jaw musculature at stage 36. The upper surface has been removed over the intermandibularis muscle and Meckel's cartilage and the distal end of the jaw and tongue are shown in transverse section.

intermandibularis the proportion of purely fast to slow fibres is 2.5:1. In comparison, the tongue muscles (hypoglossus obliquus), which are also composed of fast and slow fibres, have a constant proportion of 1.6:1 fast to slow fibres along their length (data not shown).

To trace the early development of musculature within the lower jaw, and in particular the intermandibularis, mandibular primordia at various stages of development were examined and the distribution of fast and slow muscle fibres was spatially mapped by sectioning along the proximodistal axis of the primordia. For a more detailed description of the development of the musculature of the mandibular and other visceral arches see McClearn and Noden (1988).

The first time that myogenic cells could be identified was at stage 27 (5 days of development). In the most distal sections from stage 27 primordia, no myogenic cells were detected. A few small mononucleated myogenic cells were identified in sections taken from around 450 µm from the tip of the primordium (approximately half way along the primordium). At this stage, muscle has been laid down in the proximal half of the primordium, where three separate muscle masses could be identified (Fig. 3A-D). The smallest and centrally located of the three masses is the precursor of the protractor musculature, and the other two muscle masses on each side of the midline will form the intermandibularis and the other major muscles of the lower jaw (Fig. 3B,C). In all three of the early muscle masses, the fast muscle myosin antibody (LM5) labels all the muscle fibres. Slow muscle myosins were also detected with both the slow myosin antibodies (96J and 98/31). More of the myotubes in the lateral areas of the muscle masses expressed the slow MyHC, and only a few myotubes in the medial regions (Fig. 3B,C). Of the two slow myosin antibodies, 96J stains a greater percentage of the total myotubes than does 98/31 (Table 1A). Already at this earliest stage at which muscle cells can be recognised, proportionally more of the muscle myotubes in the proximal region of the muscle masses label with the fast antibody than in the distal region (1.4:1 fast to slow distally compared to 2.2:1 fast to slow proximally; Fig. 4).

By stage 28 (5.5 days), the two muscle masses on either side of the midline have begun to fuse proximally (Fig. 3E-

Table 1. The distribution of fibre types in the muscle masses and presumptive intermandibularis muscle at different stages in the development of the lower jaw

Distance from tip (μm)	Antibody			
	83B6	LM5	96J	98/31
(A) Stage 27 $n=3$				
500-550	100	110	ND	73
600-630	ND	100	66	50
700-750	100	91	69	45
(B) Stage 28 $n=3$				
430-450	ND	100	71	ND
530-550	ND	100	59	49
650-700	100	93	48	43
820-850	100	111	50	40
(C) Stage 29 $n=2$				
350-400	100	101	ND	59
460-540	100	80	ND	63
630-750	100	92	ND	52
880-950	ND	100	ND	39
(D) Stage 36 $n=3$				
500-700	100	85	ND	70
1450-1500	100	89	ND	61
2150-2200	ND	100	ND	60
2400-2500	100	109	ND	48
4000>	100	109	ND	44

The data were collected from sections taken at different distances from the tip of the primordium. Where data from 83B6 were available (labelling all skeletal MyHC's), the number of cells labelled with this antibody was made to equal 100%. If no 83B6 value was determined, the value for LM5 (fast MyHC) was taken as 100%. n =number of mandibular primordia examined, ND not determined.

H). At this stage, the most distal cells containing muscle myosins were detected at around 350 μm from the tip of the primordium. At 440 μm from the tip, the two lateral muscle masses, which at this point along the primordium are just touching, contain multinucleated myotubes with the ratio of 1.6:1 fast to slow. Further along the jaw, at around 700 μm , the lateral muscle masses are now completely fused, and there are twice as many fast as slow muscle fibres. Finally, at the proximal end of the primordium (around 850-900 μm), the proportion of fast to slow muscle fibres was 2.5:1 (Fig. 4). Table 1B shows the gradual decline in the percentage of fibres that are labelled with both slow antibodies along the proximodistal axis of the lower jaw (96J and 98/31).

At stage 29, the fibres of the developing muscle are beginning to acquire their future orientation. The large central muscle mass now splits dorsoventrally into an upper and a lower region. The presumptive intermandibularis will form from the upper region and the fibres in this area now run horizontally (Fig. 3I-L). The lower part of the large, central muscle mass splits almost immediately into two muscle masses, one on each side between the forming Meckel's cartilages. These muscle masses are composed of fibres that run the length of the mandible and will develop into the other major first arch muscles of the lower jaw (e.g. adductor mandibulae externus). At this stage, mononucleated myoblasts were detected at 300 μm from the tip of the primordium. Further proximally, between 350 and 400 μm

from the tip where splitting has taken place, the ratio of fast to slow was 1.7:1. In the presumptive intermandibularis muscle, at around 700 μm from the tip of the primordium, the ratio in this muscle mass had increased to 1.9:1 fast to slow and, by the proximal end of the primordium (950-1000 μm), the ratio of the fast to slow had reached 2.5:1 (Fig. 4, Table 1C).

Fig. 4 shows the proportions of fast and slow fibres along the length of the primordium at the developmental stages investigated, and in the lower jaw at 10 days of development. Between stages 27 and 29, the muscle is being laid down along the proximodistal axis of the lower jaw. Between stages 29 to 36, there appears to be amplification of the established pattern.

Innervation and muscle development in the lower jaw

The nerves that innervate the muscles of the mandibular primordium arise from the trigeminal ganglion. By stage 36 the innervation patterns are well established (Fig. 5D). Two nerves can be seen clearly on each side of the lower jaw, one running down the outside of Meckel's cartilage and the other main nerve above Meckel's cartilage and innervating the intermandibularis.

To investigate the development of innervation in relation

Fig. 3. Transverse sections through the lower jaws at several developmental stages. Column (A-M) low power wax sections through the mandibular primordia (A-I) and lower jaw (M), stained with Mallory's. Column (B-N) immunofluorescence sections of the early mandibular muscle masses (B-J) and of the intermandibularis muscle (N), labelled with the fast MyHC-specific antibody LM5. Column (C-O) immunofluorescence sections of the muscle masses of the mandibular primordia (C-K), and the intermandibularis muscle (O), labelled with the slow MyHC antibody 98/31. The last column (D-P) are diagrammatic representations of the wax sections in (A-M) and show the areas of muscle development. Stage 27 (A-D), muscle is first detected within the central portion of the primordium. The boxed area in (A) shows the area in the two immunofluorescence sections (B,C). The three separate muscle masses can be clearly seen in the sections, both the fast (B) and slow (C) MyHCs are present. Stage 28 (E-H), the two lateral muscle masses have fused across the midline of the primordium, the upper muscle mass extends into the developing tongue (F,G). Boxed area in E shows the region in F and G. Stage 29 (I-L), the muscle masses begin to split into the individual muscles of the lower jaw and to assume their future fibre orientation. J and K from the region indicated in I show the presumptive intermandibularis muscle across the upper part of the section, with fibres running horizontally. The lower portion of the muscle mass on either side has fibres that are cut end on as they run along the proximodistal length of the primordium. After 10 days of development (M-P), the intermandibularis muscle (m) is prominent spanning the Meckel's cartilages (c). The immunofluorescence sections (N,O) show the central portion of the intermandibularis muscle and, in these sections, the plane of section is oblique so that the fibres appear to have being cut in cross section. The unlabelled region in the centre of the intermandibularis is the midline raphe. The other muscles have assumed their final positions around Meckel's cartilage (M and P). The tongue muscles have split and can be seen under the entoglossum (e). Scale bars: A,E,I, 100 μm ; B,C,F,G,J,K,N,O, 20 μm ; M, 200 μm .

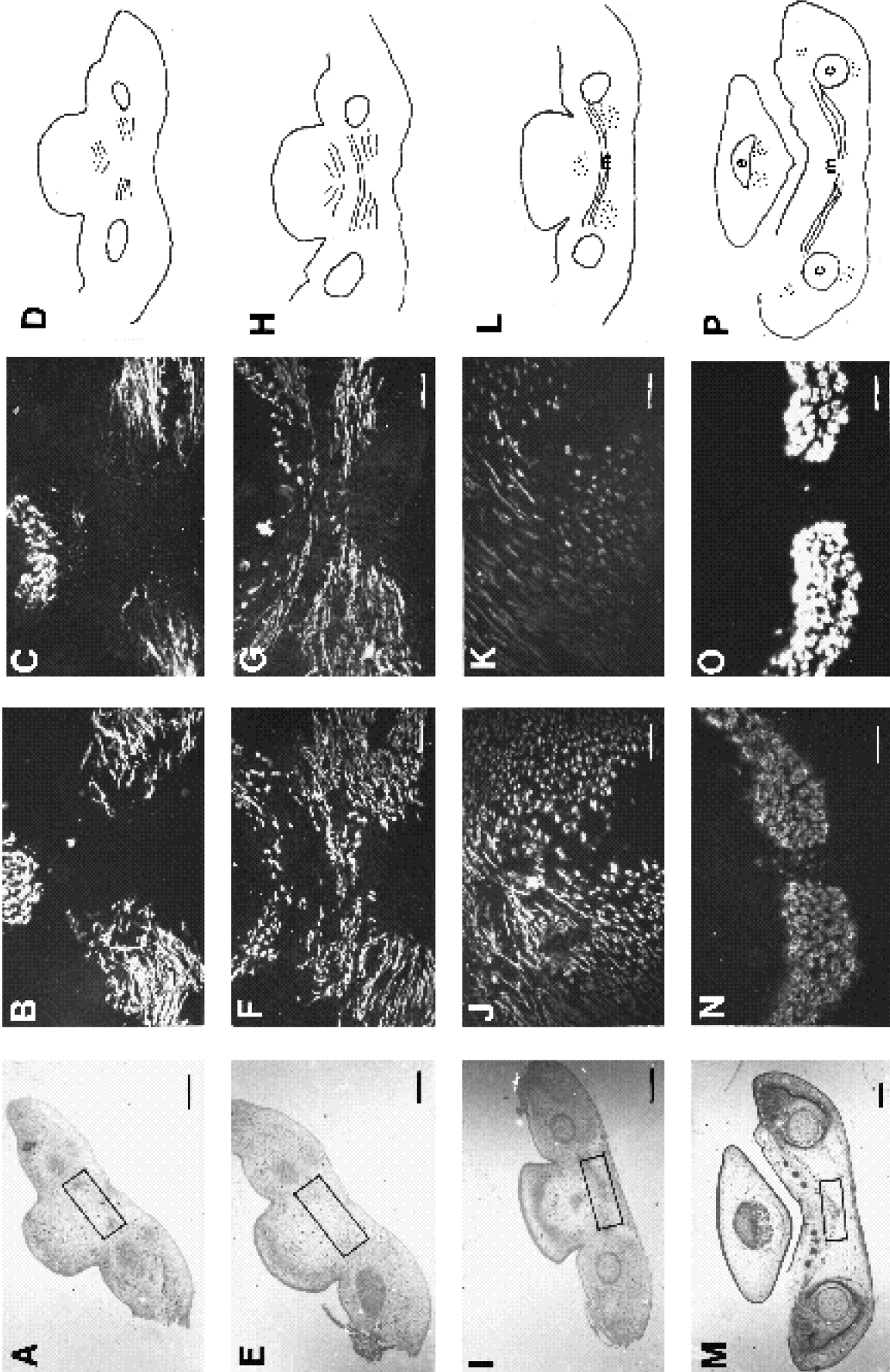


Fig. 3

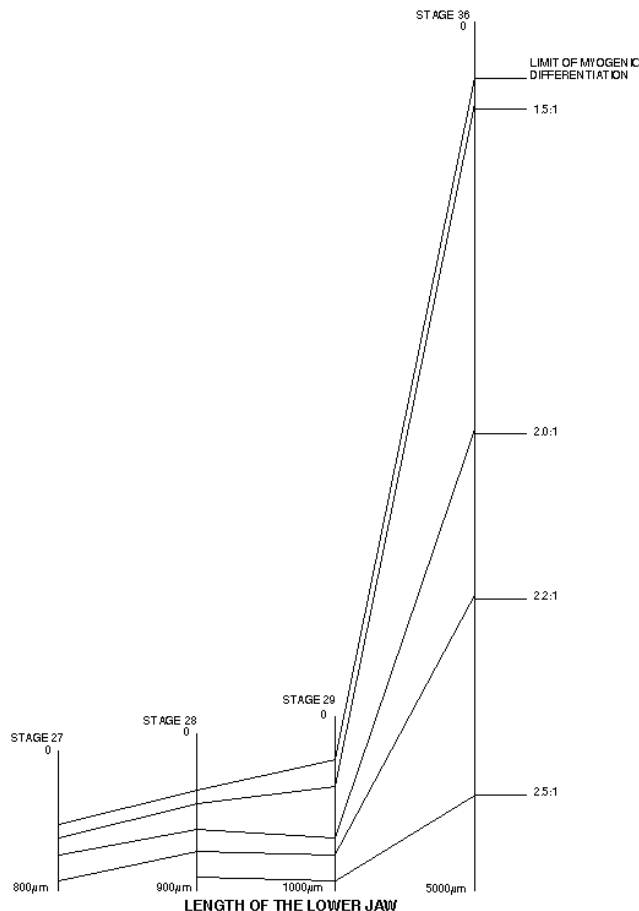


Fig. 4. Schematic maps showing the proportions of fast and slow fibres in the developing muscle along the proximodistal axis of the mandibular primordium (stage 27-29), and in the intermandibularis muscle at stage 36. The length of the primordium or lower jaw is represented by the vertical lines. Distal is designated as 0 and the distances (in μm) along the proximodistal axis of the primordia and lower jaw are measured from this.

to muscle development, the presence of nerves was examined at earlier stages. Nerves can be identified within the mandibular primordium at stage 27. These nerves are in close proximity to the areas where muscle can be seen to be differentiating (Fig. 5A). At stage 28, the nerves have progressed further into the primordium and innervate the proximal region of the muscle masses, but there appears to be no nervous innervation of the most recently differentiated muscle at the distal tip of the primordium (Fig. 5B,C).

To assess the effect of altering the innervation on the subsequent fibre type distribution during early stages of development, mandibular primordia were transplanted to a new site. The right wing bud of stage 22 chick embryos was removed and the tip of the mandibular primordia from either stage 20 or 24 embryos was pinned to the stump of the wing. In grafts from both stages, two rods of cartilage formed and an almost normal distal half of a lower beak developed (Fig. 6A,B). The grafts that developed from stage 20 mandibular primordia contained structures such as

tongue cartilage, whereas in grafts taken from stage 24 primordia only more distal structures were formed, with just the tip of the tongue developing. In both sets of grafts, muscle was observed under crossed polarising filters. When the grafts were sectioned, a nearly normal pattern of muscle development was seen, with a well-formed intermandibularis muscle, and other muscles running down the outside of Meckel's cartilage and muscles in the tongue-like structure (Fig. 6C,D). When quail mandibular primordia were grafted to the chick limb bud site, the resulting outgrowth, including the muscles were derived from the quail tissue (Fig. 6G,H). Cryosections of chick grafts labelled with the muscle antibodies showed that the proportions of fast and slow muscle in the distal region of the intermandibularis muscle were similar to those observed in the normal lower jaw at stage 36, with 1.5:1 fast to slow. More proximally in the grafts, at the level equivalent to the mid region of the lower jaw, there are twice as many fast muscle fibres as there are slow. The lower jaw that developed from the grafted primordia generally contained nerves. However, unlike the normal jaw, the four main nerves that innervated the grafted primordium ran down the centre of the jaw between the Meckel's cartilages (Fig. 5J).

The myogenic capacity of regions of the mandibular primordia in culture

To investigate the distribution of potentially fast and slow myogenic cells prior to muscle differentiation in vivo, mandibular primordia at stages 20 and 24 were dissected into distal and proximal regions. These regions were disaggregated into single cells and placed in micromass cultures in serum-free medium which promotes myogenic differentiation. In such cultures grown for 2.5 days, only single myoblasts differentiate (see also Ralphs et al., 1989).

Cultures of the distal region of stage 20 embryos had twice as many myogenic cells as the proximal region cultures as judged by labelling with the universal skeletal muscle myosin antibody (83B6). In the distal cultures, 2.2 times as many muscle cells labelled with the fast MyHC antibody (LM5), as labelled with the slow MyHC antibody (98/31). Whereas in proximal cultures, 2.9 times as many fast as slow cells were identified (Figs 7A, 8A).

At stage 24, the distal cultures contained over 7 times the number of myogenic cells found in the proximal cultures. This represents a marked increase in the number of potentially myogenic cells found in the distal region at stage 24 compared with stage 20. In contrast, proximally, the number of muscle cells had remained unchanged (Fig. 9). Despite the increase in number of muscle cells in the distal cultures, proportions of fast and slow muscle cells were similar to those obtained at stage 20 (2.2:1 as many fast as slow muscle cells). The proximal cultures at stage 24 had proportions of fast and slow MyHC-positive cells that had increased slightly from the earlier stage (3.2:1 compared with 2.9:1 Figs 7B, 8B).

Cultures were also taken from stage 28 mandibular primordia, in which myogenic cells had already started to differentiate in vivo (Fig. 2 E-H). In micromass cultures taken from the tip at stage 28, very few myogenic cells differentiated. Nevertheless the proportions of fast versus slow myosin-positive cells was still 2.3:1. In proximal cultures,

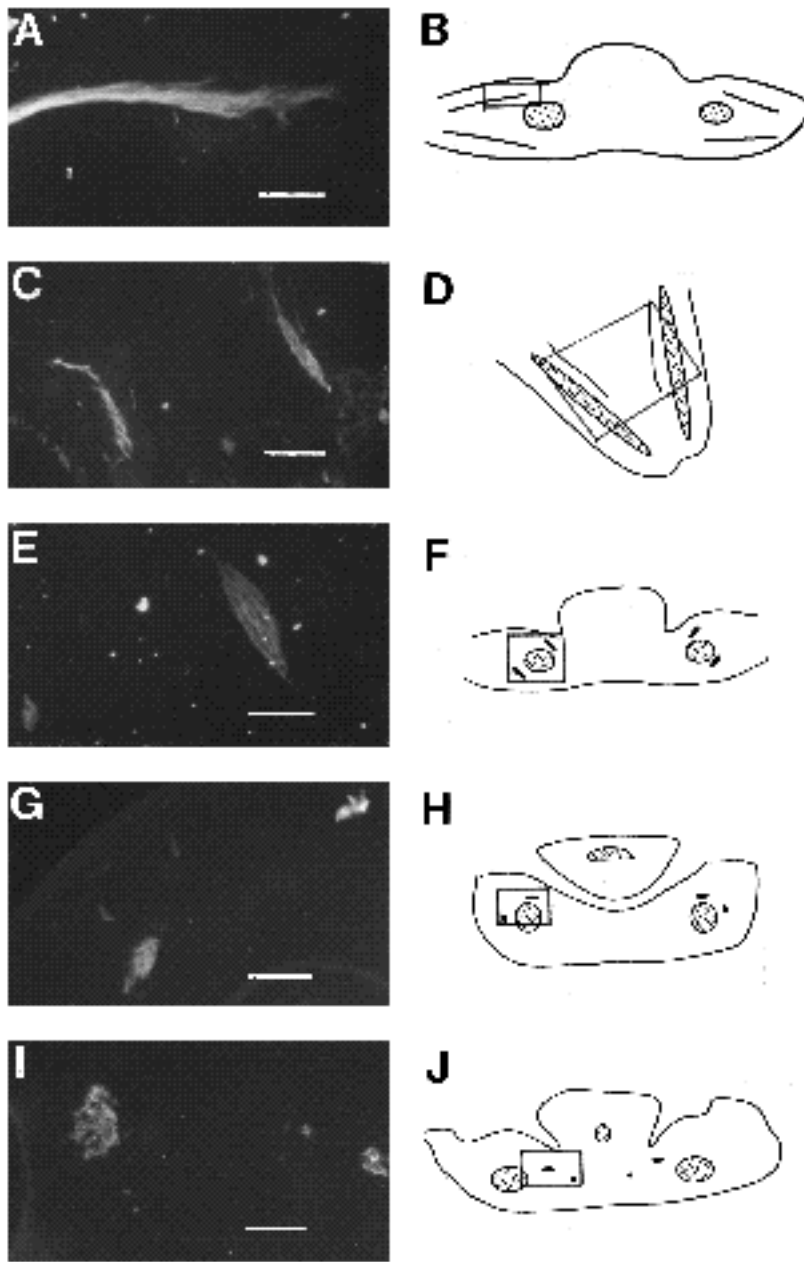


Fig. 5. Immunofluorescence labelling by the $68 \times 10^3 M_r$ neurofilament antibody, sections are cut transversely except for C, which is sectioned horizontally. (A,B) At stage 27 the nerves have entered the mandibular primordium, A is the region shown in the boxed area of B and shows the left upper nerve as it enters the primordium. (C,D) At stage 28, the nerves have progressed into the zone of differentiating muscle. C is taken from the boxed area in D and clearly shows the upper nerves running down the primordium. The boxed area in F represents the position of the nerves that can be seen in the stage 29 primordium (E). The primordium is well innervated and minor nerves branch off the main nerves to innervate the muscles. At 10 days (stage 36, see box area in H for the position of the nerves shown in G) the main nerves are above and lateral to the Meckel's cartilages. (I,J) The altered innervation in a grafted mandibular primordium, the nerves are medial to the Meckel's cartilages (box region in J shows the area in I). Scale bars A, 50 μm ; C, 20 μm ; E, 20 μm ; G, 150 μm and I, 100 μm .

the total number of myogenic cells again had not risen from the values of earlier stages, and the proportions of fast and slow have altered little with 3.5:1 fast to slow labelled muscle cells. The cultures of the mid region and from the tongue show the greatest concentration of myogenic cells and a proportion of fast to slow muscle cells of 2.4:1 (Figs 7C, 8C).

DISCUSSION

Development of muscle within the lower jaw

The intermandibularis muscle of the lower jaw of the chick embryo contains a mixture of myogenic cells, some of which elaborate both fast and slow myosins, and others that elaborate just the fast myosins. In recent studies using the

same panel of antibodies in embryonic chick leg muscles, the same two populations were seen (Williams et al., 1992). The fast antibody (LM5) labelled all the muscle fibres until about day 14, when it is restricted to the fast fibres. The slow antibody (98/31) labelled fibres that would become the future slow fibres later in development.

In the intermandibularis, there is a graded distribution of cells that synthesise fast myosins with a higher proportion of these cells proximally. This pattern reflects mainly the distribution of primary myotubes, since secondary fibres only begin to appear from stage 34 (Fredette and Landmesser, 1991). However, if the pattern is maintained in the secondary fibre population as it is in the iliotibialis and iliofibularis muscles, this may have a physiological significance. In areas of the jaw where the intermandibularis

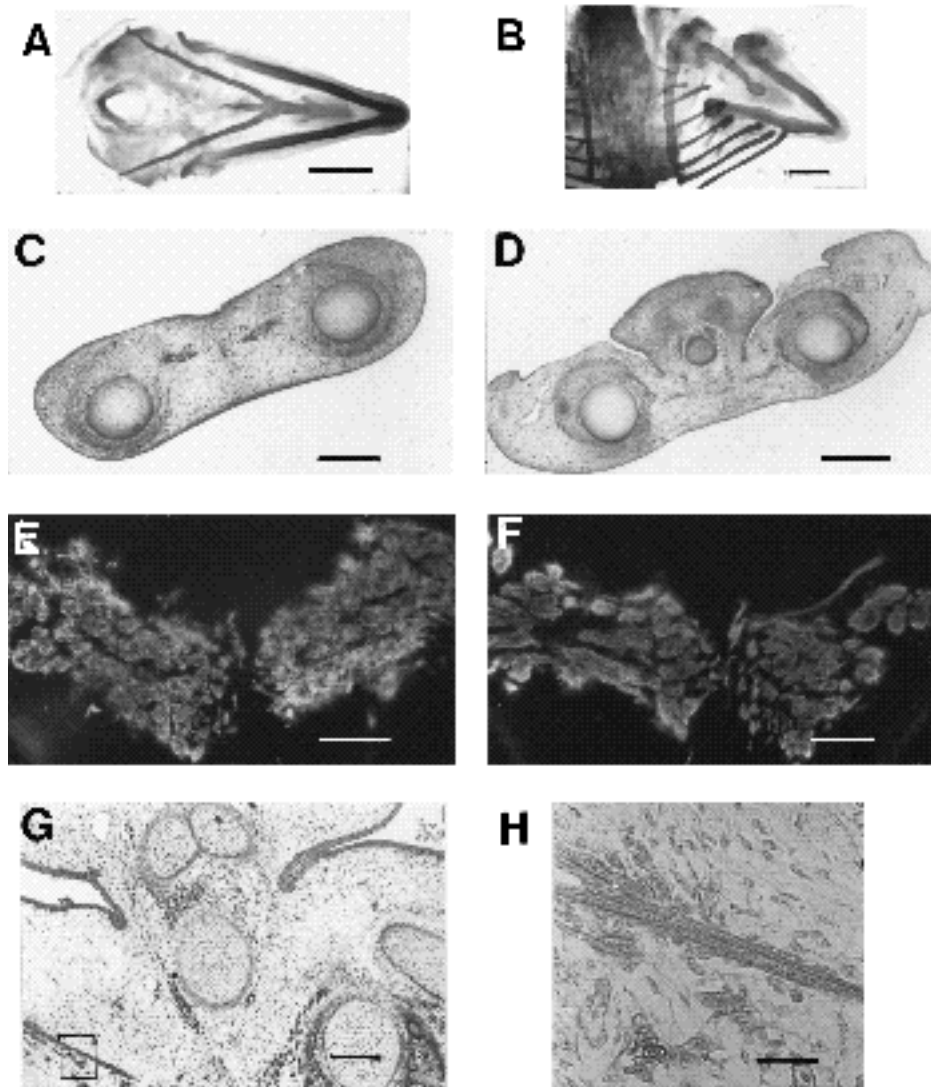


Fig. 6. (A) Normal whole-mount specimen of a 10 day (stage 36) chick lower jaw, the two Meckel's cartilages can be seen as well as the entoglossum and other hyoid cartilage elements. (B) Whole mount of a stage 24 mandibular primordium tip grafted to a chick wing bud fixed at 6 days later. Two Meckel's cartilages have developed within the grafts. The cartilage down the centre of the graft appears to have arisen from fusion of part of the humerus of the host limb with an element of tongue cartilage. (C,D) Transverse sections of the grafted mandibular primordium shown in B. (C) A distal section clearly showing that the intermandibularis has developed. (D) Proximally the intermandibularis is still present as are other muscles lateral to the Meckel's cartilage elements and within the tongue in this section. (E,F) Labelling with the fast (LM5, E) and the slow (98/31, F) muscle antibodies show the intermandibularis, with fibres running across the mandibular primordium. In this example the midline raphe is smaller than in Fig. 3 N and O. (G,H) The quail grafted mandibular primordia generally displayed more complete tongue cartilage structures, as can be seen in G. (H) The box region shown in G and the muscle fibres of the intermandibularis can be clearly seen to be of quail origin. Scale bars A and B, 1.5 mm; C and D, 200 μ m; E and F, 30 μ m; G, 200 μ m and H, 30 μ m.

muscle is involved with movements of the tongue, there is a higher proportion of the purely fast myoblasts. In the distal regions of the intermandibularis muscle that are more concerned with supporting the tongue, there is a higher proportion of the slow myoblasts.

During development of the lower jaw, the first muscle-specific myosins are detectable at stage 27 *in vivo*, with both fast and slow MyHCs being synthesised. As soon as the muscle is first laid down, a graded distribution of fast and slow fibres can be detected, with fewer fast muscle cells in the distal portion of the muscle compared to the proximal regions. It would seem that the basic topographical distribution of fast and slow fibres is established during the early period of muscle formation, appearing as soon as muscle begins to differentiate. The individual muscles of the lower jaw develop by a complex process involving both fusion and then splitting of the muscle masses on either side of the midline. This can be contrasted to muscular development within the forearm region of the wing. In the limb, the muscles develop from separate muscle masses in

the dorsal and the ventral regions which split into the individual muscles.

Differentiation of myogenic cells in culture

Cell culture provides a way of analysing the number of potentially myogenic cells that are available to construct the muscles, assuming that the culture conditions do not affect the types of myoblasts that differentiate. The data indicate that both fast and slow muscle cells are present in the early mandibular primordium, at least 2 days before the first muscle differentiates *in vivo*. The two populations could be detected in the cell cultures from embryos as early as stages 20 and 24. However, myogenic precursors are present within the primordium well before this. For example, Jacobson and Fell (1941) noted that myogenic cells were present at stage 13/14 (2 to 2.5 days).

Miller and Stockdale (1986) also found that both fast and slow myosin-positive myoblasts could be identified in cultures from 3-6 day avian limbs, before and during the appearance of the muscles. They found three distinct muscle cell lineages; 60-90% of the myoblasts were fast, 10-40%

were in a mixed fast/slow lineage and 0-3% formed the slow lineage. In contrast, the results reported here did not reveal a population of purely slow myogenic cells.

By dividing different regions of the mandibular primordia, it was found that potentially myogenic cells are concentrated distally. In distal micromass cultures from mandibular primordium of stages 20 and 24, many times more muscle cells differentiate than in the cultures from the proximal regions of the same stages. The primordia appears to lay down structures from cells at the tip. When the distal tip of the mandibular primordia is grafted to the limb bud a large outgrowth appears and 50% or more of the normal lower jaw is able to develop.

There is a large increase in the number of potentially

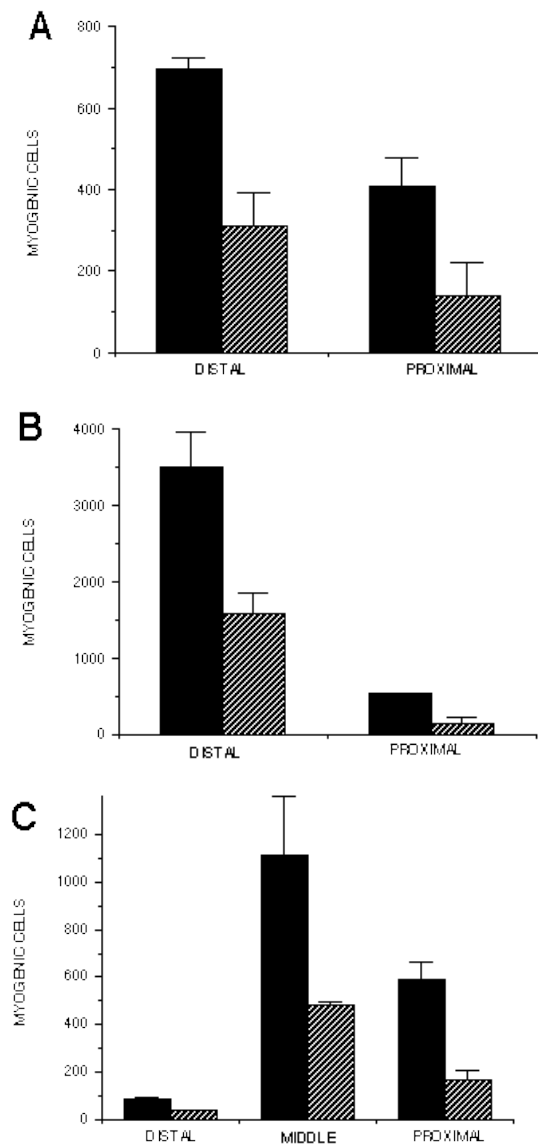


Fig. 7. Histograms of the number of myogenic cells (plus standard deviation), counted in micromass cultures from different regions of the mandibular primordia at stages 20, 24 and 28. Note the different scales. (A) Stage 20, (B) stage 24 and (C) stage 28. Solid bars LM5 (fast) and hatched bars 98/31 (slow), $n=4$ for stages 20 and 24, for stage 28 $n=2$.

myogenic cells at the tip of the primordium between stages 20 and 24. This enhancement of the myogenic cells at the distal tip of the mandibular primordium could be the result of migration of muscle cells into this region. However, the number of myogenic cells in the proximal region remains constant between stages 20 and 24. Therefore it seems more likely that proliferation of myogenic precursor cells leads to the increase in myogenic cells within the distal region of the mandibular primordium. It is interesting that the proportions of fast and slow in the distal cultures is remarkably constant despite the large increase in the total number of myogenic cells.

At stage 28, muscle has already begun to differentiate within the primordium *in vivo* and the single cells that differentiate into muscle in the cultures provide an indication of the remaining reservoir of undifferentiated myoblasts within the primordium. The number of myogenic cells at the tip of the primordium is greatly reduced from that seen at the two earlier stages. There are still potentially fast and slow muscle cells available indicating that the pool of myogenic cells that are potentially fast or slow is maintained throughout the developmental period. When the tip of stage 28 primordia is grafted to a limb bud, only the distal region of the lower jaw is formed and little muscle develops (unpublished observation).

At all stages cultured, the distal portion of the mandibular primordium contained a smaller proportion of the fast myogenic cells compared to the proximal portion. This indicates that potentially myogenic cells of the fast and slow cell type are already distributed in a graded fashion before the muscle differentiates *in vivo*.

Innervation

Moody et al. (1989) showed that the motor axons of the

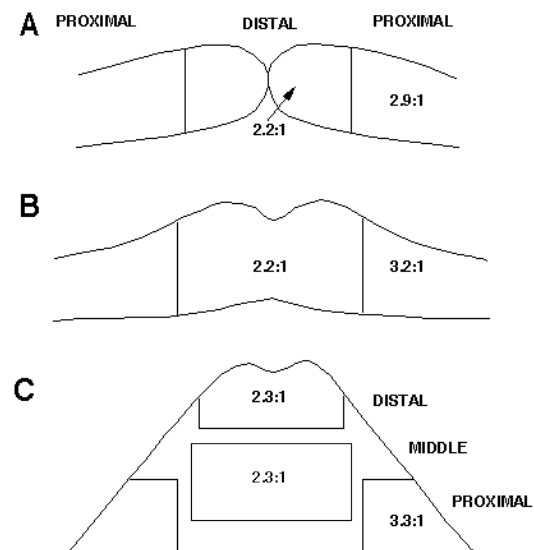


Fig. 8. Diagrammatic representations (not to scale) of the proportions of fast to slow potential myogenic cells that are present in various regions of the mandibular primordia as deduced from cells that differentiate in culture. (A) Stage 20, (B) stage 24 and (C) stage 28.

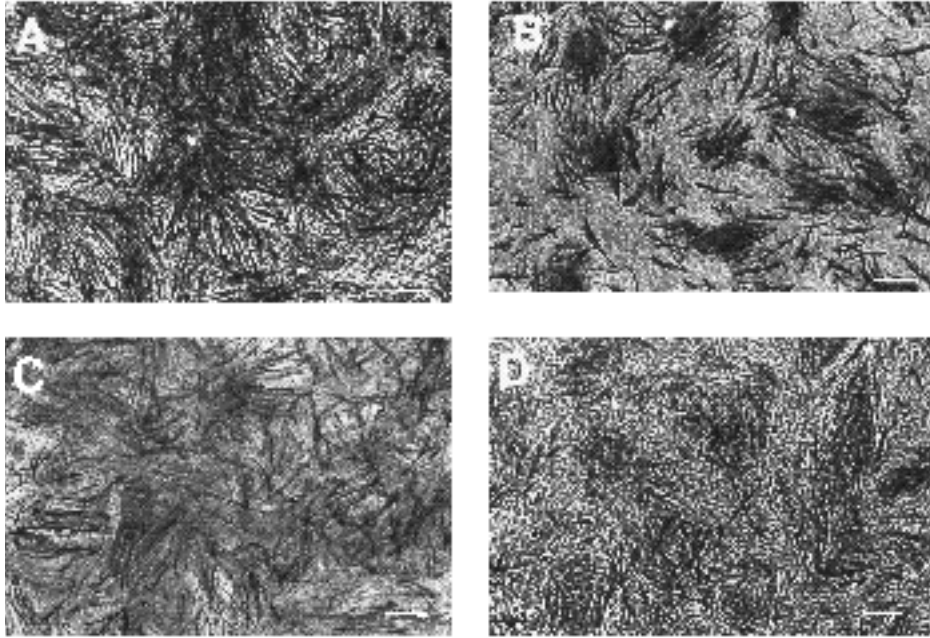


Fig. 9. Micromass cultures from stage 24 mandibular primordia. (A,B) Labelling by LM5 (fast); (C,D) labelling by 98/31 (slow). More muscle cells differentiate in distal cultures than in the proximal cultures (compare A and C with B and D). (A,C) Distal; (B,D) proximal. Twice as many fast as slow myogenic cells differentiate, whereas in the proximal cultures there are over three times as many fast as slow. Scale bars, 100 μm .

trigeminal ganglion are attracted to the mandibular muscle mass from very early in development; these pioneer motor axons are associated with the muscle mass by stage 20 (4 days). In the study reported here, the relationship between muscle differentiation and innervation was investigated as the primordium grows out. It appeared that the mononucleated myoblasts at the most distal tip of the differentiating intermandibularis muscle are not innervated by the motor axons, although innervation begins shortly after the myoblasts fuse forming primary myotubes. Moreover, in the absence of nerves, both fast and slow cells differentiate in the cultures. In addition a role for specific nerves in establishing the early differentiation into fast and slow fibres can be ruled out. In mandibular primordia grafted to the limb bud site, which are innervated by foreign motoneurons, the normal pattern of fast and slow muscle fibre distribution is seen. A similar conclusion has been reached by studies on limb muscles. Laing and Lamb (1983) grafted stage 18 and, 19 wing buds to sites of excised leg buds and showed that even though the innervation was derived from foreign motoneurons, the potentially myogenic cells in the transplanted limb bud were able to produce normal wing muscles and fibre patterns. It seems therefore, that, in the embryonic avian limb, the synthesis of both classes of myosins is possible without any specific nervous innervation. In the early limb, treatments that prevent normal neuromuscular function do not affect the differentiation of fast and slow muscle fibres (Crow and Stockdale, 1986; Weydert et al., 1987).

The independence of the early muscle fibre pattern on the source of innervation should be contrasted with mature muscle. Denervation of mature muscle often results in atrophy and loss of fibre staining characteristics (Drachman, 1964; Rubinstein and Kelly, 1978), while changing the pattern of innervation often leads to an alteration of the fast and slow isoform expression in neonatal and adult muscles (Buller et al., 1960; Streter et al., 1976).

How does the arrangement of fibre types arise in the developing muscle?

The mandibular primordium contains a pool of myogenic cells that are available to be recruited into muscles. Both fast and slow muscle cells have been identified within the primordium, even at early stages of development when the primordium is an undifferentiated mesenchymal bud. These fast and slow potentially myogenic cells are already distributed along the proximodistal axis very early in development in a graded fashion within the primordia so that they mirror what will be found in the intermandibularis muscle. However, a precise comparison shows that the newly assembled intermandibularis muscle contains a lower proportion of fast myoblasts than the pool of myogenic cells. It is not clear whether there is selective recruitment of cells into the developing muscles, or whether all the potentially myogenic cells are used. A disparity in the proportions of fast and slow muscle cells that differentiates in the cultures and in the early muscle is very clearly seen when the musculature of the tongue is analysed. In cultures taken from the tongue region at stage 28, the ratio of fast to slow was 2.3:1, and yet the muscles in the tongue itself contain a ratio of 1.6:1. It would be interesting to examine the arrangement of fibre types in muscles that develop following experimental manipulations that change the muscle patterning.

The maps showing the distribution of myogenic cells and the proportions of fast and slow along the proximodistal axis during lower jaw development show that the muscle develops in two stages. First the muscle is assembled in a proximodistal sequence and the cellular pattern is established. There is a progressive amplification of the initial proximodistal gradient in the proportions of fast and slow fibres, from 1.5:1 distally, and 2.2:1 proximally in the muscle masses, to 1.5:1 at the distal end of the intermandibularis muscle rising to >2.5:1 in the proximal intermandibularis. This could involve differential proliferation

of muscle cells, a change in isoform expression, or the differential recruitment of fast myoblasts from the remaining reservoir of potential muscle cells. In the second stage of muscle development, interstitial growth occurs and the pattern of fast and slow fibres is maintained.

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