Development 107, 743–750 (1989)
Printed in Great Britain © The Company of Biologists Limited 1989

The origin of secondary myotubes in mammalian skeletal muscles: ultrastructural studies

M. J. DUXSON[1], Y. USSON[2,*] and A. J. HARRIS[2]

[1]*The Neuroscience Centre and Departments of Anatomy and* [2]*Physiology, University of Otago Medical School, PO Box 913, Dunedin, New Zealand*

* Present address: Equipe de Reconnaissance des Formes et de Microscopie Quantitative, CERMO, Université Joseph Fourier, BP 53X, 38041 Grenoble Cedex, France

Summary

The distribution of secondary myotubes and undifferentiated mononucleated cells (presumed to be myoblasts) within foetal IVth lumbrical muscles of the rat was analyzed with serial section electron microscopy. In all myotube clusters for which the innervation zone was located, every secondary myotube overlapped the end-plate region of the primary myotube. No secondary myotubes were ever demonstrated to occur at a distance from the primary myotube innervation zone. This indicates that new secondary myotubes begin to form only in the innervation zone of the muscle. Some young secondary myotubes made direct contact with a nerve terminal, but we cannot say if this is true for all developing secondary myotubes. Myoblasts were not clustered near the innervation zone, but were uniformly distributed throughout the muscle. Myoblasts were frequently interposed between a primary and a secondary myotube, in equally close proximity to both cell membranes. We conclude that specificity in myoblast–myotube fusion does not depend on restrictions in the physical distribution of myoblasts within the muscle, and therefore must reflect more subtle mechanisms for intercellular recognition.

Key words: skeletal muscle, development, serial section reconstruction, myoblasts, myotubes, rat foetus.

Introduction

This is one of a series of papers that examine the early stages of formation of mammalian muscle to gain further insight into the mechanisms by which the numbers of myotubes are determined during embryonic and foetal development, and to see if adult muscle fibres may be derived from different cell lineages. These include ultrastructural studies (Duxson and Usson, 1989, and this article), the examination of patterns of expression of myosin isoforms throughout development (Harris *et al.* 1989*b*) and a description of nuclear birth-dating experiments (Harris *et al.* 1989*a*), as well as earlier physiological and structural studies (Ross *et al.* 1987*a*,*b*; Wilson *et al.* 1988).

Skeletal muscle cells are generated in two stages (Kelly and Zacks, 1969; Ontell and Kozeka, 1984; Ross *et al.* 1987*a*). Primary myotubes form first and define the future muscle by extending its full length and attaching to both tendons. Secondary myotubes form on the surface of primary myotubes beneath the basal lamina, the primary acting as a 'cellular skeleton' (Kelly, 1983) to guide the longitudinal growth of the secondaries, which eventually attach to the muscle tendon (Duxson and Usson, 1989) and separate laterally from the primary myotube with the appearance of basal lamina between the two myotubes.

In addition to the rather abrupt appearance of primary myotubes as compared to the progressive appearance and growth of secondary myotubes, other differences exist between the two classes of myotube. In rats (Ross *et al.* 1987*a*) and mice (Ontell and Kozeka, 1984), there is a lag of about two days following generation of primary myotubes before secondary myotubes start to form; there are characteristic differences in the positions of the myotube types within cell clusters and in their size; and developing secondary myotubes can be distinguished by the deep cytoplasmic ridges they insert into primary myotubes in their zones of attachment (Duxson and Usson, 1989). Primary and secondary myotubes also differ in their reaction to manipulations during development. Secondary myotubes are reduced in number or absent in denervated or paralysed muscles (Harris, 1981*a*; McLennan, 1983; Ross *et al.* 1987*b*) and their number is sensitive to environmental variables such as severe maternal undernutrition (Wilson *et al.* 1988). In contrast, the number of primary myotubes appears to be firmly fixed, and insensitive to experimental manipulations.

This paper is particularly concerned with the control

of secondary myotube formation, and the role the muscle innervation plays in this control. In a serial section electron microscopic study of a small mammalian muscle, the IVth lumbrical muscle of the rat hindlimb, we determine the place of origin of secondary myotubes, their physical relationship to the muscle innervation, and study the distribution of myoblasts along the surfaces of primary and secondary myotubes. In the following papers (Harris *et al.* 1989*a*,*b*), this information is correlated with the results of nuclear birthdating experiments and studies of the distribution of myosin isoforms within myotube types, to see if we can distinguish different populations of myoblasts contributing to the cell lineages of primary and secondary myotubes.

A further analysis of data from the serial reconstruction study has been presented separately (Duxson and Usson, 1989).

Materials and methods

Rats (white Wistar) were mated by coupling overnight in wire-bottomed cages, and pregnancies dated by the presence of a copulation plug at 9:00 h the following morning (E0).

The serial reconstruction data presented here come from a single E21 IVth lumbrical muscle, which was serially and semiserially sectioned across its entire cross-sectional area, from midbelly through to tendonous insertion.

Electron microscopy

Details of electron microscopic preparation were exactly as reported in Duxson and Usson (1989).

Briefly, foetuses were fixed by perfusion through the heart with fixative containing 1% paraformaldehyde and 1% glutaraldehyde in 145 mM-Hepes buffer. Dissected muscles were immersed in cold fixative for a further 2 h before processing and embedding for ultra-thin sectioning.

The serial section study commenced at the mid-belly of the muscle, near the point of nerve entry, and continued through to the tendon. The entire cross-section of the muscle was serially (section thickness 90 nm) or semi-serially (section interval 2.7 μm) sectioned, for a total length of 400 μm. If the commencement point is designated as zero along the longitudinal axis of the muscle, then the sectioning proceeded as follows; 0–10 μm serial sections, 10–105 μm semi-serial, 105–160 μm serial sections, 160–220 μm semi-serial, 220–400 μm serial sections.

Single or serial sections were collected on single-slot formvar-coated grids and viewed with a Phillips 410 electron microscope.

Analysis

Analysis proceeded at two levels. Briefly, from the entire cross-section of the muscle, four adjacent myotube clusters were followed in a 2.7 μm spaced series of electron micrographs that extended from endplate zone to insertion of the primary myotubes, a total length of over 400 μm. One of these clusters was reconstructed using the program for Macintosh computer, described in Duxson and Usson, 1989. The second, lower resolution analysis used an approximately 20 μm-spaced series of photomontages which included about one third of the total muscle cross-section (32 myotube clusters) and extended for 200 μm from the beginning of the section series. A 20 μm section interval was chosen as being smaller than the length of most mononucleate cells, making it unlikely that any cell associated with a muscle bundle would be missed. Each primary myotube in this region was numbered and traced through the series, and each cell associated with it (attached or separated secondary myotubes and myoblasts) recorded. The position of the endplate on the primary myotube, where present within the sectioned length, was identified. Sections intermediate in the series were examined as necessary to confirm the precise location of endplates. If the identity of a cell profile was in doubt, appropriate sections were re-examined in the electron microscope.

Results

Relationship of secondary myotubes to the innervation zone

Secondary myotubes were distinguished from primary myotubes on the basis of the greater size and more advanced cellular development of primaries; the characteristic insertion of young secondary myotubes onto primary myotubes (see Duxson and Usson, 1989); and the relationship of larger secondary myotubes to the cell cluster (larger secondary myotubes often were independent of the cluster at the muscle midbelly, but tended to return within the common basal lamina in more distal regions).

Fig. 1 shows a schematic representation of the distribution of secondary myotubes within the 32 myotube clusters analysed. The sectioned region included the innervation zone (indicated by a circle) for 20 of these clusters. From these 20 clusters with known innervation sites two important correlations emerge. (1) Every secondary myotube seen was associated with (i.e.: overlapped in length) the site of innervation of its cluster. (2) All neuromuscular junctions were associated with one or more (range 1–4) secondary myotubes.

In six cases, the *entire* length of a young secondary myotube was included within the length of muscle sectioned (clusters 13, 14, 23, 26, 27 and 32). These six myotubes contained few nuclei (range 2–5), and were presumably recently formed multinucleate cells. In all cases, the myotubes overlapped the endplate region of the primary myotube, although the endplate was often not centrally located along the length of the secondary myotube.

In all 32 clusters analysed, there was no example of a secondary myotube which was demonstrated to have no overlap with the innervation zone of its cluster. These results strongly suggest that formation of secondary myotubes is initiated only in the vicinity of muscle innervation.

Synaptic relationship of secondary myotubes to nerve terminals

An important question is whether young secondary myotubes must be directly contacted by a synaptic nerve terminal. The present results do not show whether *all* developing secondary myotubes receive direct synaptic contact, but some clearly do. Fig. 2

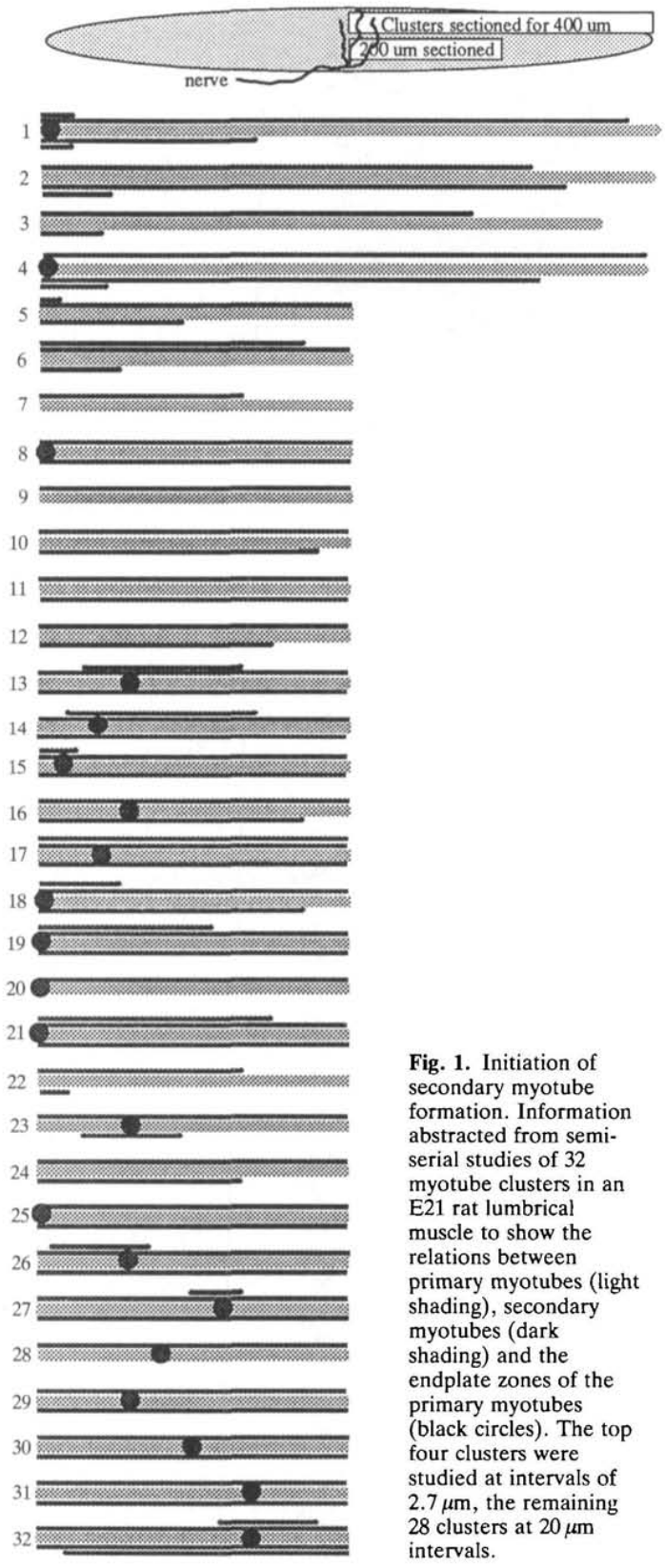

Fig. 1. Initiation of secondary myotube formation. Information abstracted from semi-serial studies of 32 myotube clusters in an E21 rat lumbrical muscle to show the relations between primary myotubes (light shading), secondary myotubes (dark shading) and the endplate zones of the primary myotubes (black circles). The top four clusters were studied at intervals of 2.7 μm, the remaining 28 clusters at 20 μm intervals.

shows some examples of the innervation of secondary myotubes at different stages of growth. In Fig. 2A a bundle of axons makes multiple synaptic contacts with a primary myotube and one of these terminals extends slightly to contact a very young secondary myotube. In an adjacent myotube cluster (Fig. 2B), a single axon terminal profile makes concurrent synaptic contacts with both the primary myotube and a medium-sized secondary myotube. Fig. 2C illustrates a well-developed secondary myotube which has separated from the myotube cluster and is contacted by an axonal branch originating from the innervation zone of the primary myotube. (Fig. 2C illustrates the same cluster as Fig. 2A, but 5 μm further on.)

We have previously suggested that secondary myotubes may receive their first innervation by transfer of a differentiated nerve terminal from the innervation zone of the primary myotube (Duxson *et al.* 1986). Studies are currently underway in our laboratory to investigate whether all secondary myotubes are directly innervated from the time of their initiation, or whether synaptic contact only becomes necessary once secondary myotubes separate from the cluster.

Distribution of mononucleate cells within the muscle

The formation of new myotubes is initiated by the fusion of myoblasts, and it is possible that the availability of myoblasts might be rate-limiting for this process (Ross *et al.* 1987*b*; Wilson *et al.* 1988). We therefore wanted to determine whether there was a local concentration of myoblasts at the endplate region of the myotube clusters.

Myoblasts were identified by their lack of myofilaments, their failure to form interlocking connections with the primary myotube (Ross *et al.* 1987*a*; Duxson and Usson, 1989), and their appearance in only a single or at most two consecutive sections. Myoblasts occurring within the basal lamina of the myotube clusters ('associated' myoblasts) were recorded separately from 'non-associated' myoblasts. In the latter class, differentiated fibrocytes were easily excluded, but the count probably includes some undifferentiated cells destined to become fibrocytes.

The total number of myoblasts (associated+non-associated) was relatively constant at each section level along the 200 μm sector of muscle analysed (Fig. 3A). However, as the neuromuscular junctions were scattered along the first 140 μm of the sector, this is not a highly informative result. In an alternative analysis, the 20 clusters with identified innervation zones were aligned with respect to the neuromuscular junction, and only myoblasts associated with these clusters considered. The analysis showed no special concentration of myoblasts in the vicinity of nerve–muscle junctions (Fig. 3B; regression line $R^2 < 0.05$).

Fig. 4 presents three views of a reconstructed myotube cluster, both with and without the primary myotube, to illustrate myoblast distribution. It was notable that myoblasts frequently lay between a primary and a secondary myotube in close proximity to both, and beneath a common basal lamina (e.g. Fig. 5). Myo-

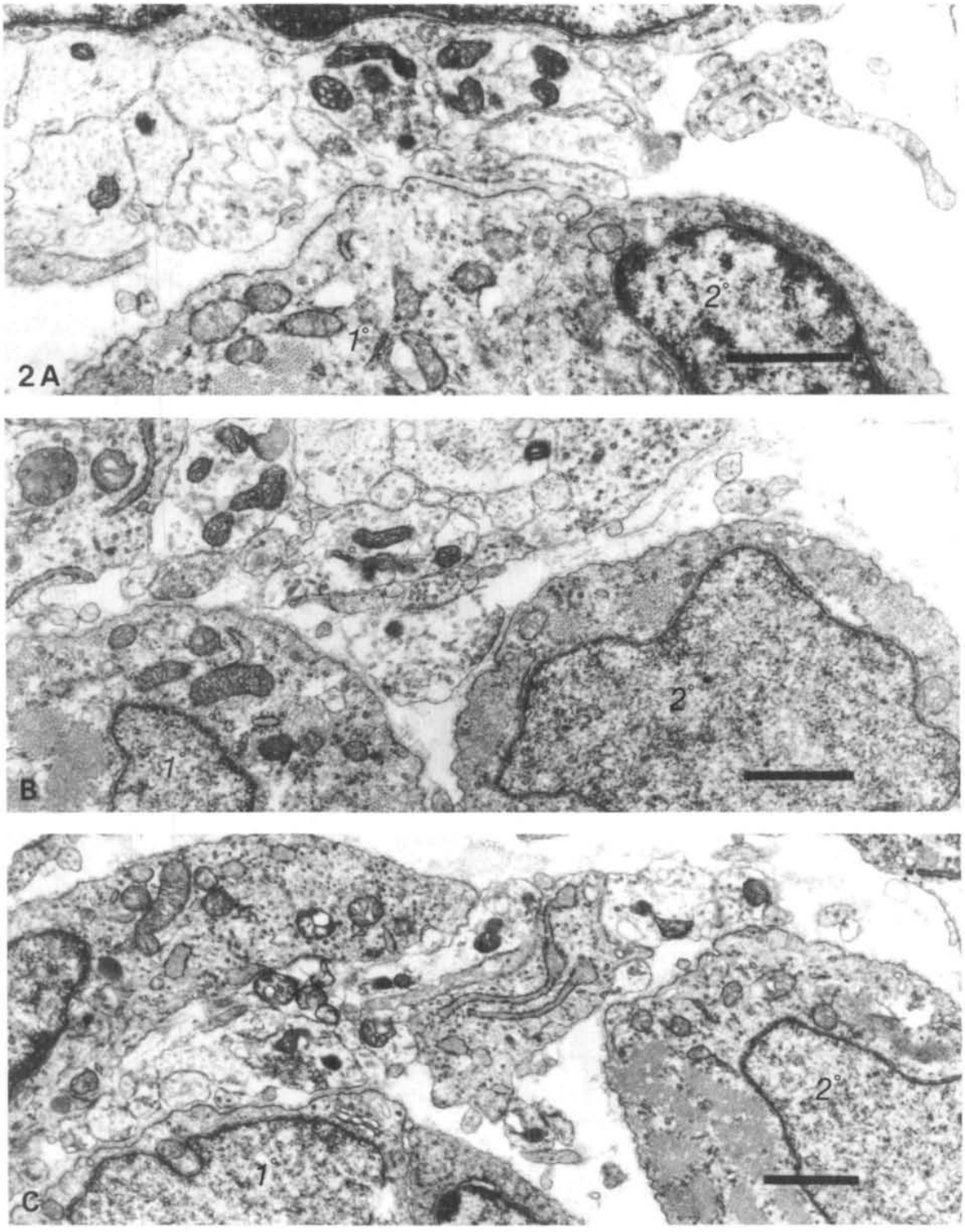

Fig. 2. Innervation of secondary myotubes. (A) A nerve terminal from the innervation zone of a primary myotube makes a relatively unspecific contact with a very young secondary myotube. (B) A more mature secondary myotube, still within the basal lamina of the primary myotube, shares a nerve terminal with the primary myotube. Clear presynaptic specializations occur in relation to both primary and secondary myotube. (C) A recently separated secondary myotube is innervated by a nerve terminal derived from the primary innervation. The axon branch leading to this terminal appears in top centre of the illustration. All pictures are from the same E21 lumbrical muscle. A and C are sections of the same myotube cluster, 5 μm apart. 1°, primary myotube. 2°, secondary myotube. Bars=1 μm.

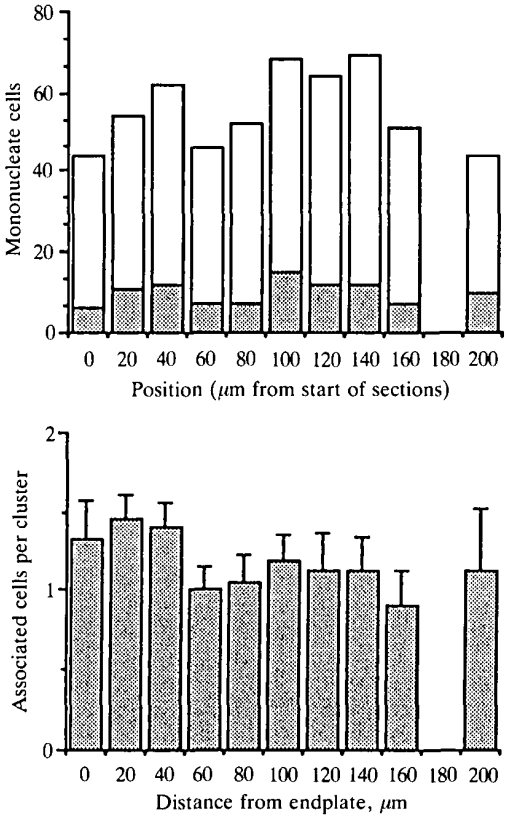

Fig. 3. Distribution of myoblasts along the length of a muscle. (A) Distribution of all myoblasts (associated myoblasts lying within the basal lamina, open bars; non-associated myoblasts, shaded bars) along a 200 μm sector of muscle; numbers of myoblasts are given in arbitrary units. The origin is at the muscle mid-belly. (B) Distribution of myoblasts closely associated with the 20 clusters having known sites of innervation, plotted with respect to their distance from the endplate zone on the primary myotube. Error bars, ±S.E.

blasts within the cluster were often present in pairs, perhaps as the consequence of a recent mitosis.

Cell coupling in E21 lumbrical muscles

All secondary myotubes formed frequent gap junctions with their parent primary myotube (Ross *et al.* 1987*a*). As the secondary myotubes matured and separated from their primary myotube, gap junctions disappeared at the midregions but persisted nearer the cell extremities. Similarly, although primary myotubes were well separated from one another in their midregion, many retained gap junctions with other primary myotubes in the region of the tendon.

Gap junctions made by mononucleated cells were particularly searched for in the series of 2.7 μm resolution sections, but very rarely seen. Every one observed was between a mononucleated cell and a secondary myotube (Fig. 5). No gap junctions were seen between a mononucleated cell and a primary myotube, or between two mononucleated cells. The latter observation is in contrast to the situation observed in tissue culture, where gap junctions between myoblasts are ubiquitous (e.g.: Harris *et al.* 1971).

Gap junctions formed by mononucleated cells had less obvious membrane densities than those involving two myotubes. Nevertheless, it is unlikely that their apparent rarity resulted from their being overlooked, as a high magnification scan of the perimeter of each cell was made under the microscope at the same time as taking the lower magnification photographs for the serial reconstructions. Also, a large archive of photomicrographs from other lumbrical and sternomastoid muscles showed very few junctions involving mononucleated cells. It is possible that such junctions are shortlived and only exist briefly prior to fusion of the myoblasts (Rash and Fambrough, 1973).

Discussion

Initiation of secondary myotube formation

Secondary myotubes form only if motor innervation is both present and active (Harris, 1981*a*; Ross *et al.* 1987*b*). Although innervation is a prerequisite for secondary myotube generation, there is no absolute requirement for a nerve terminal to be present at the actual moment of formation of the myotube. Harris (1981*a*) and Ross *et al.* (1987) both showed, in different muscles (rat diaphragm and lumbrical, respectively), that new secondary myotubes continue to appear for about 1 day after destruction of synaptic terminals with the neurotoxin β-bungarotoxin.

The studies reported here, of a normal muscle taken near the peak time of formation of secondary myotubes, advance our understanding of secondary myotube formation by showing that secondary myotubes begin to form only in the region of innervation of the muscle. In contrast, myoblasts are relatively evenly distributed throughout the muscle. Thus, we conclude that myoblasts can only fuse with each other (in contradistinction to *myoblast–myotube* fusion) in regions where nerve terminals are present or (in the case of experimentally denervated muscles) have recently been present.

Two possible ways in which initiation of secondary myotube formation might be controlled are: that it depends on an inductive signal passed from a nerve terminal to a mononucleated cell to render it receptive to fusion; or that a signal conveying fusion competence is present specifically at the primary myotube endplate (either within the extracellular matrix, on the myotube surface or released from the myotube) and the role of the nerve is to stimulate mitosis of myoblasts competent to respond to this signal. In this regard, it is interesting that junctional clusters of ACh receptors are first seen in rat diaphragm late on E15 or early E16 (Harris, 1981*a*), the same time that the earliest secondary myotubes can be detected (Harris *et al.* 1989*b*). These two events may be the result of a common inductive signal from either developing motor nerve terminals or specialized endplate structures.

The possibility that nerve may stimulate the pro-

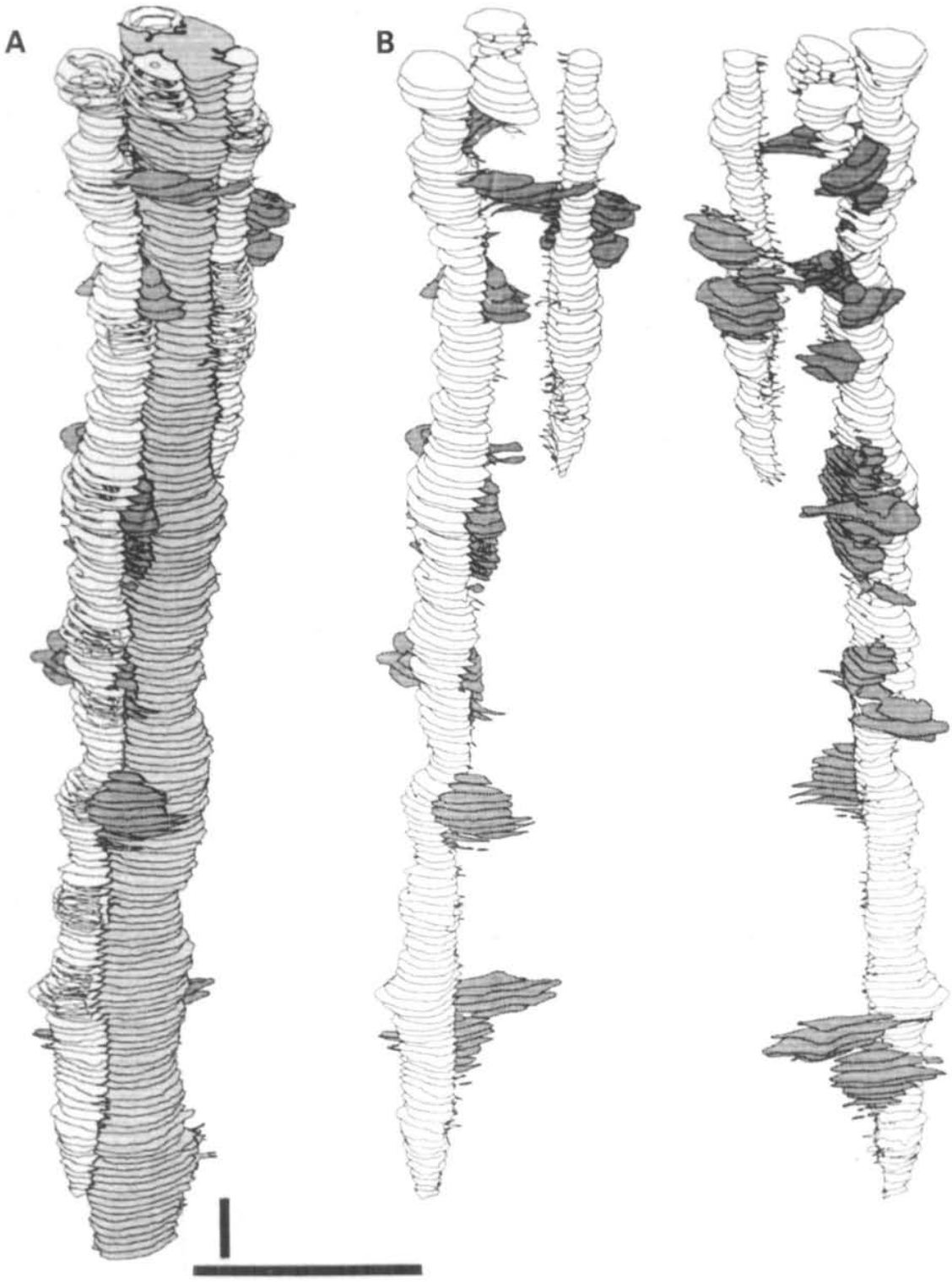

Fig. 4. Relationships between cells in a muscle cluster. (A) A computer-generated serial section reconstruction, using sections at 2.7 μm intervals, of a 400 μm long portion of a myotube cluster from an E21 rat hindlimb IVth lumbrical muscle showing the general relationships between primary and secondary myotubes and myoblasts. The cluster comprises a primary myotube (intermediate shading), 4 associated secondary myotubes (light shading) and 16 myoblasts (dark shading). Apparent holes in the secondary myotubes represent the positions of myonuclei. The horizontal scale is 4× greater than the vertical, exaggerating apparent myotube diameters. (B) Two rotated views of the reconstruction with the primary myotube removed, showing the secondary myotubes and the positions of the myoblasts, which often lie between the primary and a secondary myotube. Bars=20 μm.

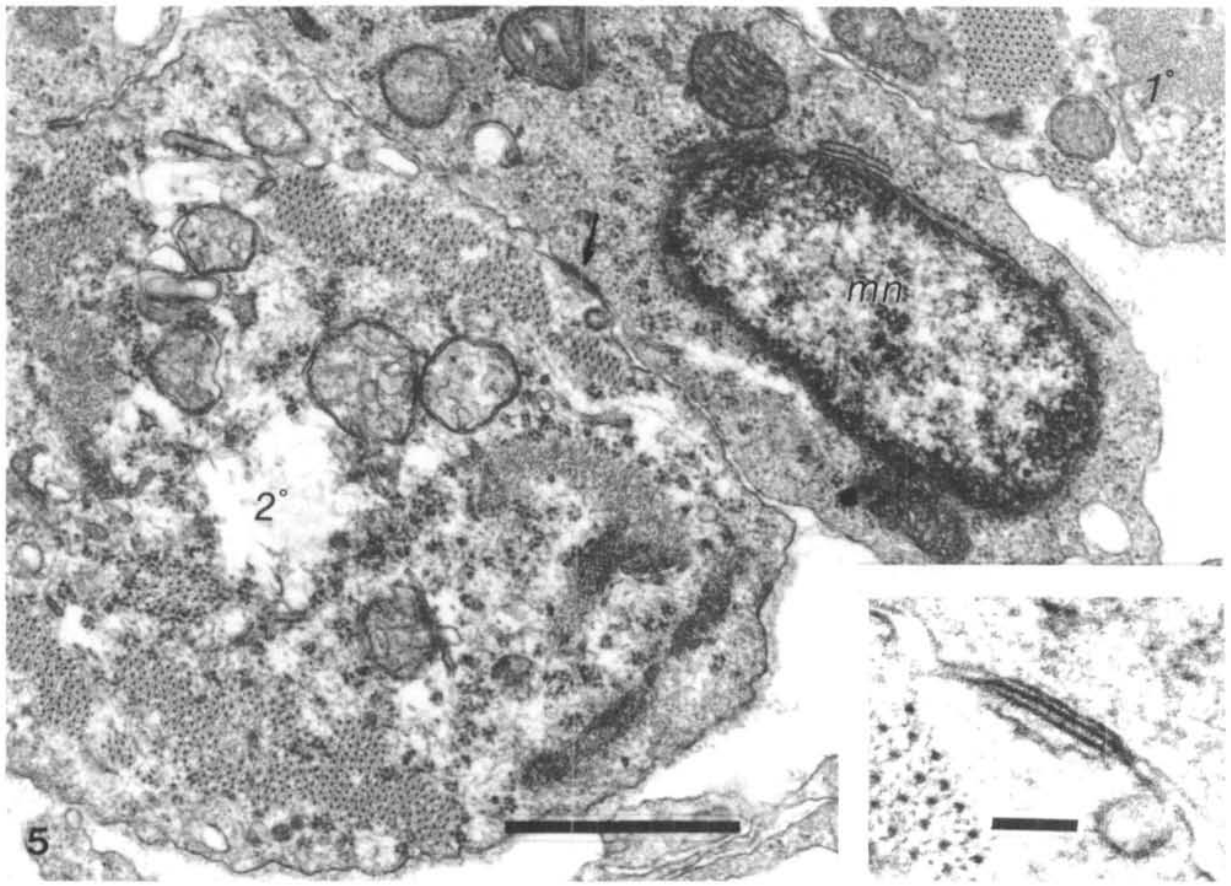

Fig. 5. Gap junction (arrow) between a secondary myotube (2°) and a mononucleate cell (mn) in an E21 lumbrical muscle. The junction is inset at the bottom right, with the viewing plane tilted by 28°. Bars: 1 μm for main picture, 0.1 μm for inset.

duction of fusion-competent myogenic cells receives support from the observations of Ross *et al.* (1987), who saw a reduction in the number of mononucleated cells in the endplate region of denervated muscles. However, the present results show there is no local accumulation of myoblasts at the endplate region of normal muscle. If myoblast proliferation is stimulated by innervation, then either the myoblasts migrate rapidly throughout the muscle or the mechanism acts uniformly along the muscle length.

Cell-type specificity in myogenic cell fusion?

In a freeze-fracture study of myoblast fusion in tissue culture, Kalderon and Gilula (1979) showed that myoblasts initially were metabolically coupled *via* gap junctions but the incidence of coupling was reduced to <50 % at the time fusion began. Fusion was initiated by the appearance of regions of membrane without intramembranous particles, and no gap junctions were seen in the fusion regions. They suggested that there is an intercellular recognition event that precedes fusion. The ability to form gap junctions may be an assay for the competence of myoblasts and myotubes to form close membrane appositions, as a possible preliminary to fusion.

At E21, when secondary myotubes in IVth lumbrical muscle are growing rapidly, we saw a few gap junctions between secondary myotubes and myoblasts. None were seen between primary myotubes and myoblasts, despite the fact that myoblasts were in equally close proximity to primary and secondary myotubes beneath a common basal lamina. This may indicate that myoblasts were specifically recognising, prior to fusing with, secondary myotubes but not primary myotubes.

No gap junctions were ever seen between myoblasts, despite the frequent observation of closely associated myoblast pairs. This observation may not be so surprising as it first seems. The results reported here suggest that myoblasts *in vivo* do not fuse with each other in any region except the endplate zone; only one fusion between myoblasts is needed for each secondary myotube formed. Thus the number of fusions between myoblast pairs will be very small beside the number of myoblast–secondary myotube fusions needed to promote growth of the myotube.

Although these correlations are interesting to consider, there is no evidence to suggest that gap junctions are an obligatory precedent to cell fusion, or that they necessarily result in fusion (Kalderon and Gilula, 1979). In fact, we noted that their most frequent occurrence in developing muscle is between myotubes, which do not fuse with one another.

In conclusion, the results reported here indicate that secondary myotubes begin to form only near the endplate region of the primary myotube; that young secondary myotubes may receive direct innervation but this is probably not obligatory during the fusion event that initiates their formation; and that there is no local concentration of myoblasts at the endplate region to explain why myoblast fusion should preferentially occur there.

This work was supported by the New Zealand Medical Research Council, the Muscular Dystrophy Association of America and the Vernon Willey Trust.

References

Duxson, M. J., Ross, J. J. & Harris, A. J. (1986). Transfer of differentiated synaptic terminals from primary myotubes to new-formed muscle cells during embryonic development in the rat. *Neurosci. Lett.* **71**, 147–152.

Duxson, M. J. & Usson, Y. (1989). Cellular insertion of primary and secondary myotubes in embryonic rat muscles. *Development* **107**, 243–251.

Harris, A. J. (1981*a*). Embryonic growth and innervation of rat skeletal muscles. I. Neural regulation of muscle fibre numbers. *Phil. Trans. Roy. Soc., Lond. B.* **293**, 257–277.

Harris, A. J. (1981*b*). Embryonic growth and innervation of rat skeletal muscles. III. Neural regulation of junctional and extra-junctional acetylcholine receptor clusters. *Phil. Trans. Roy. Soc., Lond. B.* **293**, 287–314.

Harris, A. J., Duxson, M. J., Fitzsimons, R. B. & Rieger, F. (1989*a*). Myonuclear birthdates distinguish the origins of primary and secondary myotubes in embryonic mammalian skeletal muscles. *Development* **107**, 000–000.

Harris, A. J., Fitzsimons, R. B. & McEwan, J. C. (1989*b*). Neural control of the sequence of expression of myosin heavy chain isoforms in foetal mammalian muscles. *Development* **107**, 000–000.

Harris, A. J., Heinemann, S., Schubert, D. & Tarakis, H. (1971). Trophic interaction between clonal tissue culture lines of nerve and muscle. *Nature, Lond.* **231**, 296–301.

Kalderon, N. & Gilula, N. B. (1979). Membrane events involved in myoblast fusion. *J. Cell Biol.* **81**, 411–425.

Kelly, A. M. (1983). Emergence of specialization of skeletal muscle. *In Handbook of Physiology* (L. D. Peachey, Ed.) Section 10, pp. 507–537.

Kelly, A. M. & Zacks, S. I. (1969). The histogenesis of rat intercostal muscle. *J. Cell Biol.* **42**, 135–153.

McLennan, I. S. (1983). Neural dependence and independence of myotube production in chicken hindlimb muscles. *Devl Biol.* **98**, 287–294.

Ontell, M. & Kozeka, K. (1984). The organogenesis of murine striated muscle: a cytoarchitectural study. *Am. J. Anat.* **171**, 133–148.

Rash, J. E. & Fambrough, D. (1973). Ultrastructural and electrophysiological correlates of cell coupling and cytoplasmic fusion during myogenesis *in vitro*. *Devl Biol.* **30**, 166–186.

Ross, J. J., Duxson, M. J. & Harris, A. J. (1987*a*). Formation of primary and secondary myotubes in rat lumbrical muscles. *Development* **100**, 383–394.

Ross, J. J., Duxson, M. J. & Harris, A. J. (1987*b*). Neural determination of muscle fibre numbers in embryonic rat lumbrical muscles. *Development* **100**, 395–409.

Wilson, S. J., Ross, J. J. & Harris, A. J. (1988). A critical period for formation of secondary myotubes defined by prenatal undernourishment in rats. *Development* **102**, 815–821.

(*Accepted 31 August 1989*)