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Development of myotomal cells in *Xenopus laevis* larvae

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INTRODUCTION

The physiology of embryonic amphibian muscle differs in some respects from that of adult muscle. For example, developing *Xenopus* myocytes show calcium, rather than sodium, action potentials at early developmental stages (Blackshaw & Warner, 1974). This is in parallel with observations in embryonic avian muscle (Kano, 1975) and developing nerve (Spitzer, 1979). The underlying calcium currents persist in less prominent form in adult amphibian muscle (Beaty & Stefani, 1976; Stanfield, 1977). The small size of embryonic myocytes results in larger surface–volume ratios than in adult muscle, which could allow membrane excitation to be coupled to contractile activation through extracellular calcium influx, as may operate in cardiac muscle. This would be in contrast to the situation in adult skeletal muscle which involves calcium release from sarcoplasmic reticular stores but relatively little participation of extracellular calcium influx (Costantin, 1975).

However, recent physiological experiments (Huang, 1986) have suggested that the mechanism of contractile activation closely resembles the pattern in adult muscle even in early stages (Stage 23–24, as laid down by Nieuwkoop & Faber, 1956) of embryonic development. Such a hypothesis would specifically predict the appearances of not only contractile myofilaments, but also transverse tubules and sarcoplasmic reticulum as soon as contractile activity is observed at early stages of development. The latter would be necessary if electrical activity initiated in surface membrane is to be conducted to the cell interior thereby to regulate sarcoplasmic reticular calcium release (Peachey & Franzini-Armstrong, 1983). The communication between tubular and sarcoplasmic reticular membrane is thought to require the mediation of 'triad' complexes where these membranes are geometrically close (Franzini-Armstrong, 1970, 1975; Schneider & Chandler, 1973; Chandler, Rakowski & Schneider, 1976). It would therefore also be desirable to investigate the appearance of such structures. Earlier work (Blackshaw & Warner, 1974) has provided electron microscopic appearances of some of these features. Other studies have described the differentiation of membrane systems in avian muscle *in vitro* (e.g. Ezerman & Ishikawa, 1967). Additionally it would also be appropriate to investigate whether the transverse tubular membrane communicates with the cell surface early in embryogenesis.

This paper goes on to describe the *in vivo* appearances of developing myotomes in greater detail, and through a wider sequence of embryonic stages in amphibian muscle than reported earlier, selected to correspond to those reported in the earlier physiological paper (Huang, 1986). Accordingly, the selection of myotome segments for morphological study was standardised (see Methods). Particular interest was directed at the appearance of the above membrane systems associated with excitation-contraction coupling of junctional complexes between them, and the possible

communication of tubular membrane with myocyte surface as assessed using the extracellular marker, ruthenium red.

METHODS

Matings of South African clawed frogs (*Xenopus laevis*) were induced using chorionic gonadotrophin (Organon, U.K.). Fertilized eggs were removed soon after the resulting spawning. They were left in freshly drawn tap water and kept at 15–20 °C. The frogs used were laboratory bred and reared and normally first filial to supplied (wild) stock. Embryos were staged using the external criteria of Nieuwkoop & Faber (1956). At the appropriate developmental stages, they were placed in a Sylgard-(Dow Chemical Co. USA) bottomed chamber filled with a solution of 115 mM-NaCl, 2·5 mM-KCl, 3·0 mM-$CaCl_2$, 2·0 mM-$MgCl_2$, 1 mM tricaine methanesulphonate (Sigma, UK), and 3 mM hepes at pH 7. Their developing central nervous systems were destroyed by mounting them by their cephalic ends using an A1 insect pin, so that they lay on their sides but with their dorsal edge inclined slightly upwards.

The ectoderm was then removed on both sides using fine dissecting needles under cold (fibre optic) illumination under × 40 magnification. The most anterior myotomes were identified in the dorsal part of the embryo. During development, blocks of dorsal mesoderm rotate and differentiate into myotome cells whose principal axes are parallel to that of the embryo. This process occurs sequentially along the embryo in the rostrocaudal direction. Therefore, at any given embryonic stage, the anterior somites consistently represent the most advanced phase in development (Hamilton, 1969). A fine metal needle with a bevelled cutting edge was then used to separate the anterior somites from the rest of the embryo for study.

The excised specimen was then placed in fixative (2% formaldehyde, 2% glutaraldehyde, and 0·1 M cacodylate buffer at neutral pH) for 2–3 hours, and then washed in 0·1 M cacodylate buffer, postfixed in 1% osmium tetroxide in 0·1 M cacodylate and embedded in Araldite. Tissue was sectioned at 50–100 nm. Sections for preliminary light microscopy were stained using toluidine blue. Those for direct electron microscopy were stained using uranyl acetate and lead citrate.

Continuity of transverse tubules with surface membrane was evaluated by using ruthenium red (Agar Aids Ltd. U.K.) (500 ppm) staining before preparation (Luft, 1971*a*, *b*). Sections were cut to a thickness of approximately 100 nm and lightly stained with lead citrate. Transmission electron microscopy was performed using a Philips EM300 electron microscope.

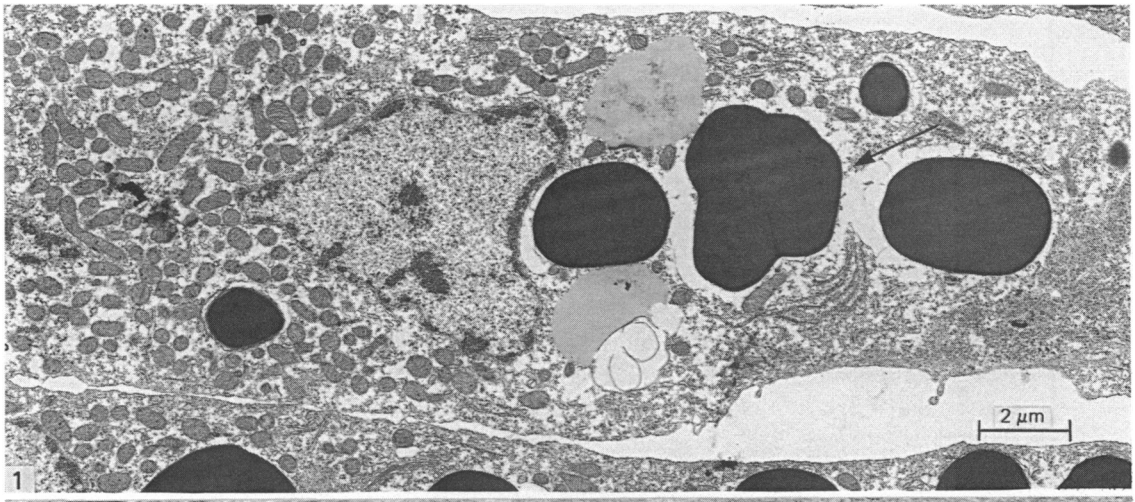
RESULTS

Embryos were studied between developmental Stages 22 and 41. At Stage 22, somites become just visible as myotomal segments located at the anterior end of the embryo (Hamilton, 1969). Mechanical activity is first detectable at Stages 24–25,

Fig. 1. Myotome from Stage 22. Little evidence of differentiation of myofibrils. Yolk platelets are arrowed.

Fig. 2. End region of myocyte from Stage 24 embryo stained with extracellular ruthenium red showing disordered myofibrils and some associated membranes. Arrow indicates membrane structures staining positively with ruthenium red.

Fig. 3. Stage 28 embryo. Fully assembled sarcomeres with transverse tubules in register. Organised thick and thin filaments. Note the appearance of triad structures.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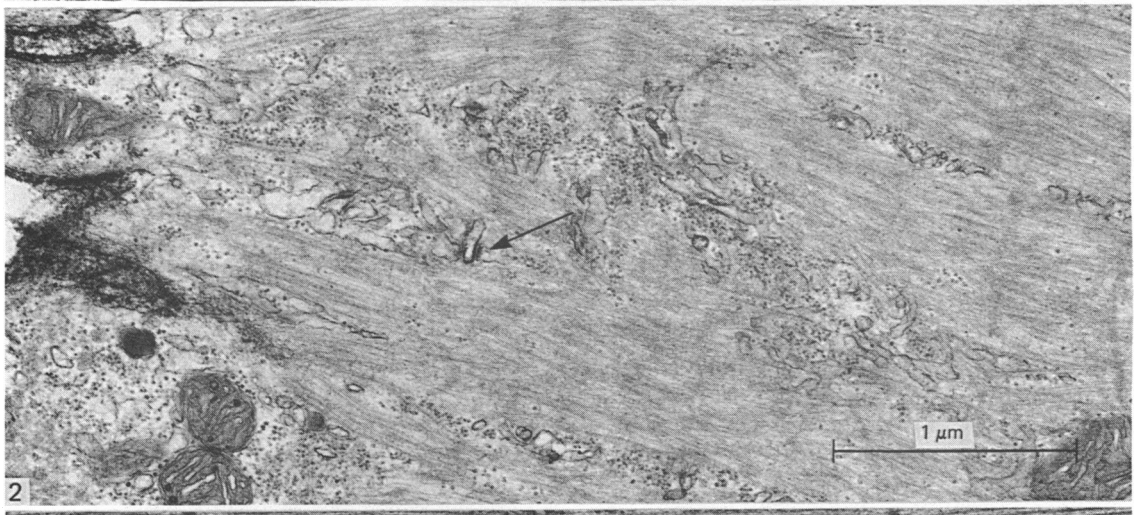
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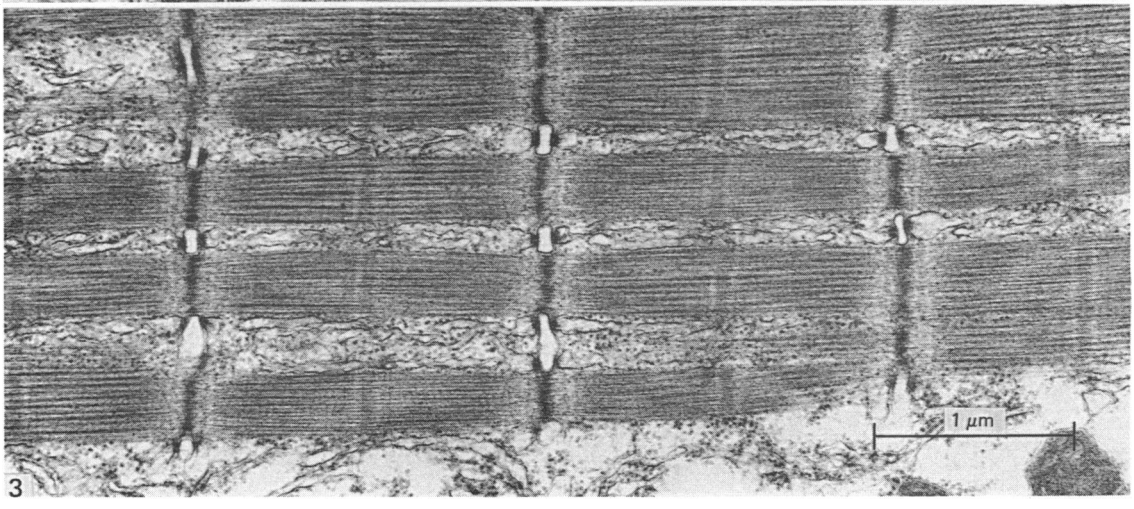
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and becomes clear-cut by Stage 28. By Stage 35, the initially slow flexing movements become more rapid. Accordingly, the stages examined here parallelled those described in the earlier physiological study (Huang, 1986) with particular reference to morphological features listed in the Introduction.

Figure 1 shows typical electron microscopic appearances of presumptive myotomal cells from early embryos at Stage 22–23. In this, and throughout all the subsequent stages examined, myotomal cells were uninucleate and discrete. They had an ordered arrangement with their long axes parallel to that of the rest of the embryo. At Stage 22–23, the cells were spindle shaped and contained numerous mitochondria, yolk platelets (arrowed) and rough endoplasmic reticulum. However, assembled thick and thin contractile filaments were absent. There was no evidence of sarcoplasmic reticular or transverse tubular membranes in a scan of 20 cells. This absence of organised structures associated with contractile activation correlates with the absence of mechanical activity in response to imposed depolarisation reported in the earlier work (Huang, 1986).

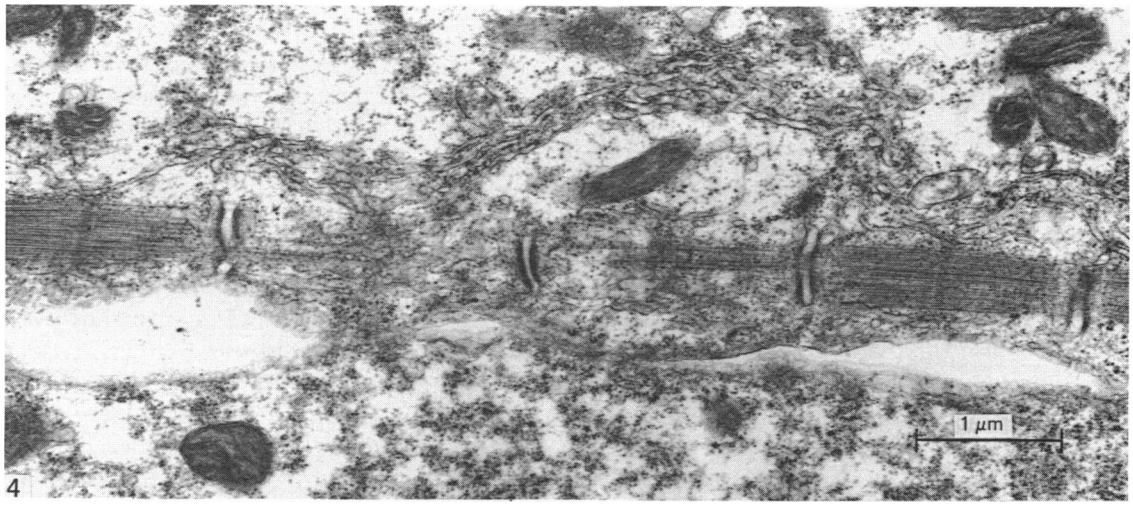
Specialisation into a contractile function was first observed in anterior somites of embryos between Stages 24–25. About 60% of the cells observed then still did not show any evidence of myofilament ultrastructure or the associated membrane systems. However, thick and thin filaments were observed close to the cell surface in the remaining myotome cells. Figure 2 shows a section stained with ruthenium red and lead citrate. It shows myofilaments in a disordered arrangement. Nevertheless, thick and thin filaments could be differentiated in these clusters, particularly in other sections stained with both uranyl acetate and lead citrate. They tended to run parallel to the direction of the surface membrane. Membrane-bound lumina could be seen with such clusters. A few of these formed complexes closely resembling the 'triad' structures observed in mature muscle. They became easier to demonstrate at later stages. Even at such early stages of development, some of these presumably transverse tubular membrane systems in the process of formation, appeared stained positively with ruthenium red (arrowed). Under such conditions, this is believed only to stain membrane in communication with the cell exterior and so acts as an extracellular marker. Thus, surface membrane stained positively and some of this positive staining also occurred in the presumptive tubular membranes.

More advanced stages in sarcomere formation were also demonstrated at Stage 24–25. This was consistent with the onset of contractile activity observed in the physiological experiments (Huang, 1986). Such appearances became the rule in later stages. For example, by Stage 28, the clusters of unorganised thick and thin filaments had disappeared. All the myofilaments were organised instead into regular arrays of thick and thin filaments, and clear A and I bands and H and Z lines, well known in adult muscle, were present (Fig. 3). Each strand of contractile myofilaments was separated from its neighbours by cross sections of transverse tubular membrane. Figure 3 also shows that junctions between tubular and sarcoplasmic reticular membrane systems were prominent and occurred early in development, then usually occurring in 'triad' complexes. 'Dyad' complexes were rarely if ever observed. Triad complexes occurred at regular intervals coincident with the Z lines. Where there was

Fig. 4. Development of myofibrils in relation to the tubular system in longitudinal section in myocyte from Stage 28 embryo.

Fig. 5. Ruthenium red staining of tubular, and surface, but not sarcoplasmic reticular membranes in a sarcomere in the process of formation. Stage 28 embryo.

Fig. 6. Stage 41 embryo. Myocyte at low power. Cell filled with myofilaments.



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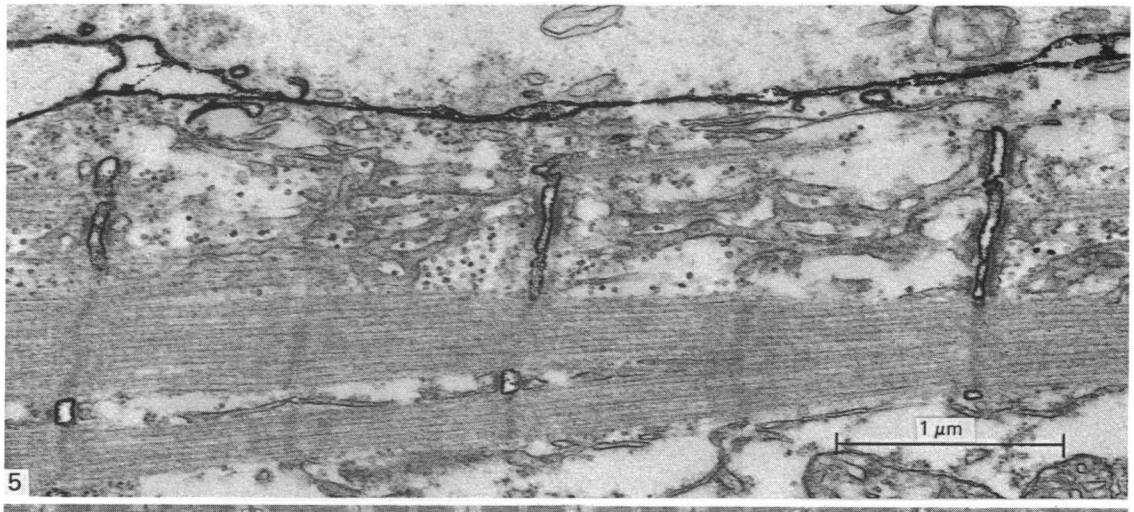

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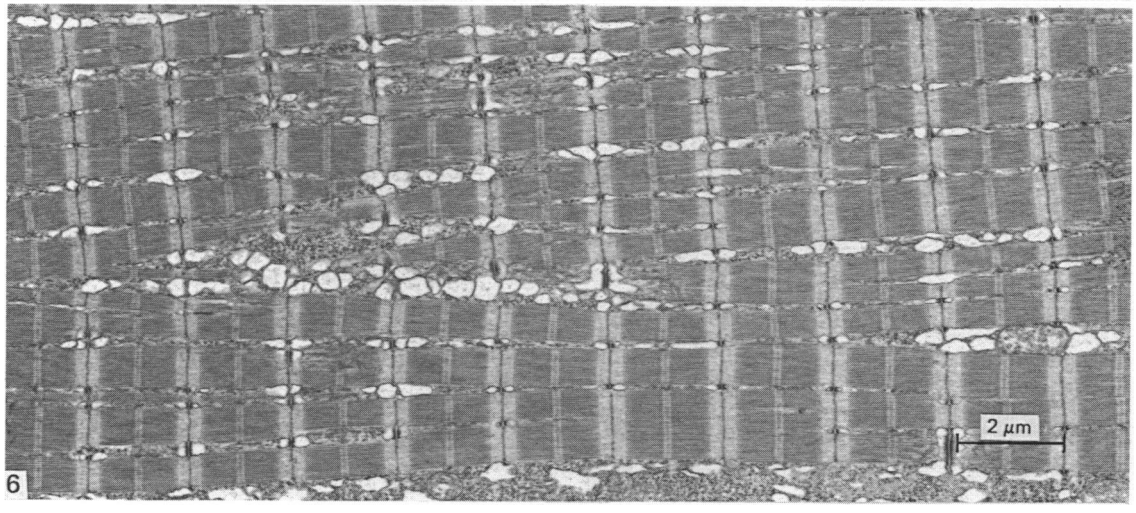

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more than one row of myofilaments the Z lines of such adjacent strands were 'in register' with each other.

Where myofilaments were observed, they were invariably associated with cross sections of transverse tubules. In contrast, repeating membrane structures representing developing tubular system were sometimes observed, frequently in association with sarcoplasmic reticular membrane formations in the absence of, or the minimal development of, organised myofilaments. Figure 4 illustrates transverse tubules in longitudinal section, which particularly illustrates establishment of a repeating pattern of tubules, as myofilaments were relatively less prominent. Furthermore, even early in sarcomere formation, it is likely that tubular membrane is continuous with surface membrane but not with sarcoplasmic reticular membrane. Thus Figure 5 shows a ruthenium red-stained section of a sarcomere in the process of formation where the (regularly repeating) tubular system stained positively, but the intervening sarcoplasmic reticular membrane did not. This confirms that transverse tubular membrane should be able to conduct surface electrical changes to the 'triad' structures during excitation-contraction coupling (*cf.* Huang, 1986).

By Stage 35, the myofilaments were aligned parallel to, and extended throughout, the whole length of the cell. Completed myofilaments appeared first to be laid down directly beneath the surface membrane, particularly along the outer margin of each myocyte as also illustrated in Figure 3. Usually 2–3 layers of myofilaments were observed with I and Z bands in register. The associated transverse tubular membranes continued to communicate with surface membrane at the later stages examined (28, 35, 41, 42) as indicated by ruthenium red staining. With these advancing stages, myotome cells increased their content of contractile organelles. By Stage 41–42 a pattern very similar to the adult morphology was reached, with cells filled with contractile filaments associated with tubular and sarcoplasmic reticular membranes (Fig. 6).

DISCUSSION

This short paper examines the structural features of developing *Xenopus laevis* myocytes early in their development. Blackshaw & Warner (1976) first showed electron micrographs of some developing organelles in these cells in connection with their characterisation of gap junctions connecting them, concentrating in particular at Stages 22–23. Such gap junctions ensure that despite the small size of each myocyte, the developing muscle mass on each side of the embryo behaves as an electrical syncytium, not unlike the situation in adult smooth muscle. However, each individual myocyte would have a large surface–volume ratio. Additionally these cells are known to fire calcium rather than sodium action potentials at early stages of their development (Blackshaw & Warner, 1974; Kano, 1975). All these features suggest a participation of extracellular calcium entry in activation of early embryonic muscle, a situation similar to that in smooth muscle.

However, physiological experiments have suggested that the mechanism of contractile activation, even early in development, closely resembles that in adult skeletal muscle as soon as such functions can be detected. In these experiments contraction altered sharply as membrane potential was altered by perfusing solutions containing different potassium concentrations. It was not affected by preventing extracellular calcium entry either using Ca^{2+}-free solutions or with calcium channel block by Mn^{2+}. Activation of adult and embryonic muscle was similarly affected by lyotropic ions and by caffeine and tetracaine (Huang, 1986).

The present experiments have examined the development of the associated anatomical features in *Xenopus* myocytes at Stages between 21–42. These correspond to stages described in the earlier physiological study (Huang, 1986). Similar cells were selected for study: these were obtained from the anterior 2–3 somites in each embryo. Particular attention was paid to development of transverse tubules and their possible communication with either surface membrane or sarcoplasmic reticulum.

Using the above criteria, thick and thin filaments and signs of developing (but not organised) tubular membranes appeared close to Stage 24. Even by Stage 28, the adult pattern had begun to differentiate. Highly developed transverse tubular (T) and sarcoplasmic reticular (SR) membrane systems could be particularly demonstrated in instances where their organisation preceded that of thick and thin filaments into A and I bands. The periodic spacing of tubules occurred even early in development. Even at such stages, ruthenium red markers suggested that tubular membrane, but not sarcoplasmic reticulum, was continuous with cell surface. 'Triad' complexes occurred early, and were not preceded by simpler (e.g. diad) forms of T:SR associations. These morphological results relate well to physiological findings at like stages (Huang, 1986), and are consistent with earlier morphological observations (Blackshaw & Warner, 1976).

Contractile activity becomes more marked and vigorous beyond Stage 24. This correlated not so much with the degree of myofibril differentiation as to a quantitative increase in the amount of contractile material in each cell. Myofilament strands extended the whole length of each cell by Stage 33, although only one or two such strands then occurred in each longitudinal section. However, myofilaments increased in number until the entire cell appeared filled by Stage 41. The findings are therefore consistent with rapid assumption of adult morphological as well as physiological features of contractile activation in *Xenopus*, and a close correlation between development of structure and acquisition of function. They also emphasise the order in which development of organised sarcomeres takes place. In particular, the first signs of thick and thin filaments and of a transverse tubular system appeared beneath the surface membrane. Similarly, myofilaments were always associated with tubular and sarcoplasmic reticular membrane systems, but the latter could often be observed without the former. Finally, the filling of myocyte cells with myofilaments took place from beneath the surface of each cell inwards. These observations are consistent with a role of the tubular system in organising condensation of myofilament strands; such speculation may merit further testing.

SUMMARY

Electron microscopic appearances of *Xenopus laevis* axial myotome cells were examined through Stages 22–41 (Nieuwkoop & Faber, 1956). Differentiated contractile structures were not observed in myotome cells at or before Stage 22. At Stages 24–25, myofibrils appeared both as disordered strands in association with tubular membranes, and in different degrees of assembly into ordered sarcomeres. By Stage 28, all contractile organelles observed were organised into sarcomeres. The latter extended the length of each myocyte by Stage 35. Myofibrils were initially laid down adjacent to the cell membrane in each myocyte close to its lateral surface. They filled most of each cell by Stage 41. Membrane structures known to be associated with contractile activation in adult muscle appeared early in development. Transverse tubular, and sarcoplasmic reticular membranes, and 'triad' complexes could be demonstrated from the outset of sarcomere formation at Stage 24. In places,

establishment of a regular repeating tubular system appeared to precede myofilament organisation. Examination of sections stained with ruthenium red during preparation suggested that tubular and surface membranes were continuous even at Stage 24 and all subsequent developmental stages studied here. Earlier work (Huang, 1986) has suggested that excitation-contraction coupling in embryonic muscle involves release of stored calcium in response to tubular voltage changes as in adult muscle. These findings corroborate such physiological observations.

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