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The Origin of Syncytial Muscle Fibres in the Myotomes of *Xenopus laevis* – a Revision*

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During the early stages of myogenesis in X. laevis, the primary myoblasts (of mesodermal origin) differentiate simultaneously, in each myotome, into mononucleate myotubes. At later stages mesenchymal cells appear in intermyotomal fissures and then in the myotomes between myotubes and contribute to the formation of syncytial muscle fibres. The pathway of mesenchymals cell during myogenesis was described in *X. laevis* by monitoring the incorporation of ^{3}H-thymidine. ^{3}H-thymidine was incorporated in the nuclei of mesenchymal cells in intermyotomal fissures of younger myotomes and then in those of older myotomes between the myotubes revealing the proliferation of mesenchymal cells. As expected, nuclei of differentiating mononucleate myotubes did not incorporate ^{3}H-thymidine. At later stages of myogenesis the myotubes were found to contain two classes of nuclei: large nuclei of the primary myoblasts (of myotomal origin) and smaller nuclei originating from secondary myoblasts of mesenchymal origin. TEM and autoradiographic analyses confirm that mulinucleate myotubes in *X. laevis* arise through fusion of secondary myoblasts with mononucleate myotubes.

Key words: Myogenesis, mesenchymal cells, autoradiography, amphibia.

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Myotomal myogenesis in chordates deserves particular attention because of its diversity between taxa. Chordate myotomal myogenesis proceeds from mononucleate muscle cells to multinucleate muscle fibres (KIEŁBÓWNA & DACZEWSKA 2004).

Myotomal myogenesis in the phylogenetically older anuran families Pipidae (*Xenopus laevis*, *Hymenochirus boeetgeri*) and Discoglossidae (*Bombina variegata*) is particularly important in the evolution of myogenesis. Mononucleate myotubes, appearing at early stages of myogenesis, resemble mononucleate muscle cells and represent a transitory phase in the development of multinucleate muscle fibres (KIEŁBÓWNA & DACZEWSKA 2004).

The development of monucleate muscle cells (mononucleate myotubes) has been found to be preceded by two different types of myoblast kinetics. Myoblasts of *X. laevis* and *H. boettgeri* occupy the whole length of the myotome as a result of their rotation from perpendicular to parallel to the long axis of the embryo (HAMILTON 1969; KIEŁBÓWNA 1981; YOUNG & MALACINSKI 1981; DACZEWSKA 2001). In *B. variegata* mononucleate myotubes occupy the whole length of the myotome as a result of a different process – directional growth of the myoblasts (KIEŁBÓWNA & KOŚCIELSKI 1979; KIEŁBÓWNA 1981).

In other anurans such as *Pelobates fuscus*, *Rana lessonae*, *Rana dalmatina* and *Rana temporaria*, myotomal myoblasts fuse to form multinucleate myotubes (KIEŁBÓWNA 1987; DACZEWSKA & PAŁUCKA 1999; RADICE *et al.* 1989). The beginning of myotomal myogenesis is similar in the urodeles *Ambystoma mexicanum*, *Hynobius keyserlingii* and *Triturus vulgaris* (RADICE *et al*. 1989; DACZEWSKA & KIEŁBÓWNA 2000). However SATO (1992), studying myotomal myogenesis in the Japanese newt *Cynops pyrrhogaster*, rejected this hypothesis stating that multinucleate myotubes arise by multiple mitotic divisions of the myoblast nucleus, without accompanying cytokinesis.

A common feature of all amphibians studied (i.e. *X. levis*, *H. boettgeri*, *B. variegata*, *P. fuscus*, *R. lessonae*, *T. vulgaris*) is the involvement of all myo-

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tomal myoblasts of each myotome in the process of myotube differentiation (KIEŁBÓWNA 1966; DACZEWSKA 2001; KIEŁBÓWNA 1987; DACZEWSKA & PAŁUCKA 1999; DACZEWSKA & KIEŁBÓWNA 2000). Further myotube development in *H. boettgeri*, *B. variegata*, *P. fuscus*, *R. lessonae* and *T. vulgaris* involves the mesenchymal cells which migrate into the myotomes (DACZEWSKA 2001; KIEŁBÓWNA & KOŚCIELSKI 1979; KIEŁBÓWNA 1987; DACZEWSKA & PAŁUCKA 1999; DACZEWSKA & KIEŁBÓWNA 2000). The pathway of their migration was first traced in myotomal myogenesis of *B. variegata* using ^{3}H-thymidine. Marked cells appeared in intermyotomal fissures, then some of them migrated to the myotomes, between the myotubes. These cells (secondary myoblasts) subsequently fused with the myotubes (KIEŁBÓWNA & KOŚCIELSKI 1979). TEM studies show that in *T. vulgaris* and *H. boettgeri* mesenchymal cells located in the intermyotomal fissures retain their fibroblastic properties, whereas cells which have entered into direct contact with the myotube acquire myogenic capacity (DACZEWSKA & KIEŁBÓWNA 2000; DACZEWSKA 2001). The myoblastic features of these cells are confirmed by their ability to fuse with the myotubes (DACZEWSKA 2001). Myoblasts of mesenchymal origin, at a later stage of myogenesis, are involved in the development of secondary myotubes, e.g. in *P. fuscus* and *R. lessonae* (KIEŁBÓWNA 1987; DACZEWSKA & PAŁUCKA 1999). The absence of any cells in the neighborhood of differentiating mononucleate myotubes in *X. laevis* was, in the first studies on amphibian myotomal myogenesis, a great obstacle in ascertaining the source of new nuclei appearing in the myotube at a later stage of myogenesis. Initially it was believed that the new, small nuclei in *X. laevis* arose through budding off from the large, mother nucleus of the myotube, and subsequent mitotic divisions of the small nucleus (KIEŁBÓWNA 1966). Another hypothesis was presented by MUNTZ (1975). In her opinion the source of the new nuclei appearing in the mononucleate myotube were satellite cells located in the terminal parts of the myotube. In a later paper BOUJELIDA & MUNTZ (1987) assumed that multinuclearity of myotomal myotubes in *X. laevis* resulted mainly from a multiple amitotic division of the myotube nucleus.

The fact that the mechanism of formation of multinucleate muscle fibres remains obscure, and that even authors studying the process from a genetic viewpoint are not sure which hypothesis is right (NICOLAS *et al*. 1998; CHANOINE & HARDY 2003) prompted us to re-analysis of myotomal myogenesis in *X. laevis*, presented below.

Material and Methods

Studies on myotomal myogenesis in *X. laevis* included developmental stages from early tail bud (stage 30) to the stage of free swimming tadpole (stage 48). A total of 40 specimens were examined. Development stages were determined according to the development table for *X. laevis* (NIEUWKOOP & FABER 1956). Reproduction in sexually mature males and females was induced by injecting their dorsal lymphatic sacs with 500 IU of gonadotropic hormone (Biogonadyl). The progeny was kept in aired aquaria at 20-24 ^{o}C. At early development stages the gelatinous envelopes were removed prior to fixation. For this purpose the embryos were subject to UV irradiation for several minutes, until the envelope dissolved completely, and were then immediately transferred to the fixative. Tadpoles that had left their egg envelopes were anasthesised with Tricaine (MS 222) prior to fixation.

TEM material was fixed for 24 hrs in modified Karnowsky's liquid (1% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer). The material was repeatedly rinsed in the same buffer and postfixed for 2 hrs in 1% OsO_4 in phosphate buffer, pH 7.4. After rinsing in 0.1 M phosphate buffer, the material was dehydrated in a graded alcohol series and acetone, and embedded in epoxy resin Epon 812. The Epon blocks were cut on a Reihert Ultracut E ultramicrotome. Semithin sections (0.6 μm) were stained with methylene blue in 1% borax and examined under an Olympus BHS light microscope. Ultrathin sections were contrasted with uranyl acetate and lead citrate according to the standard Reynolds (1963) method, and examined in a TEM Zeiss EM 900 at an accelerating voltage of 80 kV.

Autoradiography

For autoradiographic investigations tadpoles of three developmental stages (stages 33, 40, 45) were used. The dorsal body parts of the tadpoles (myotomes) were incubated in Stainberg's fluid containing 100 μc/ml ^{3}H-thymidne. The incubation time was from 3 to 22 h. The material was fixed in Carnoy's solution and embedded in paraffin. 5 μm sections were coated with NTB-2 (Eastman Kodak, Rochester, NY) emulsion, dried at room temperature and exposed for 10 months at 4 ^{o}C, when NTB-2 emulsion was developed.

Results

During the early stages of myogenesis in *X. laevis* (stage 30), the primary myoblasts (of mesodermal origin) occupied the whole lengths of the myotomes and were arranged parallel to the long axis of the embryo. They differentiated into mononucleate myotubes (stage 35). The presence of mature myofibrils below their plasmalemma is a manifestation of this process. The myotube nuclei were centrally located, their cytoplasm was filled with yolk platelets and lipid droplets (Figs 1, 2). Differentiation

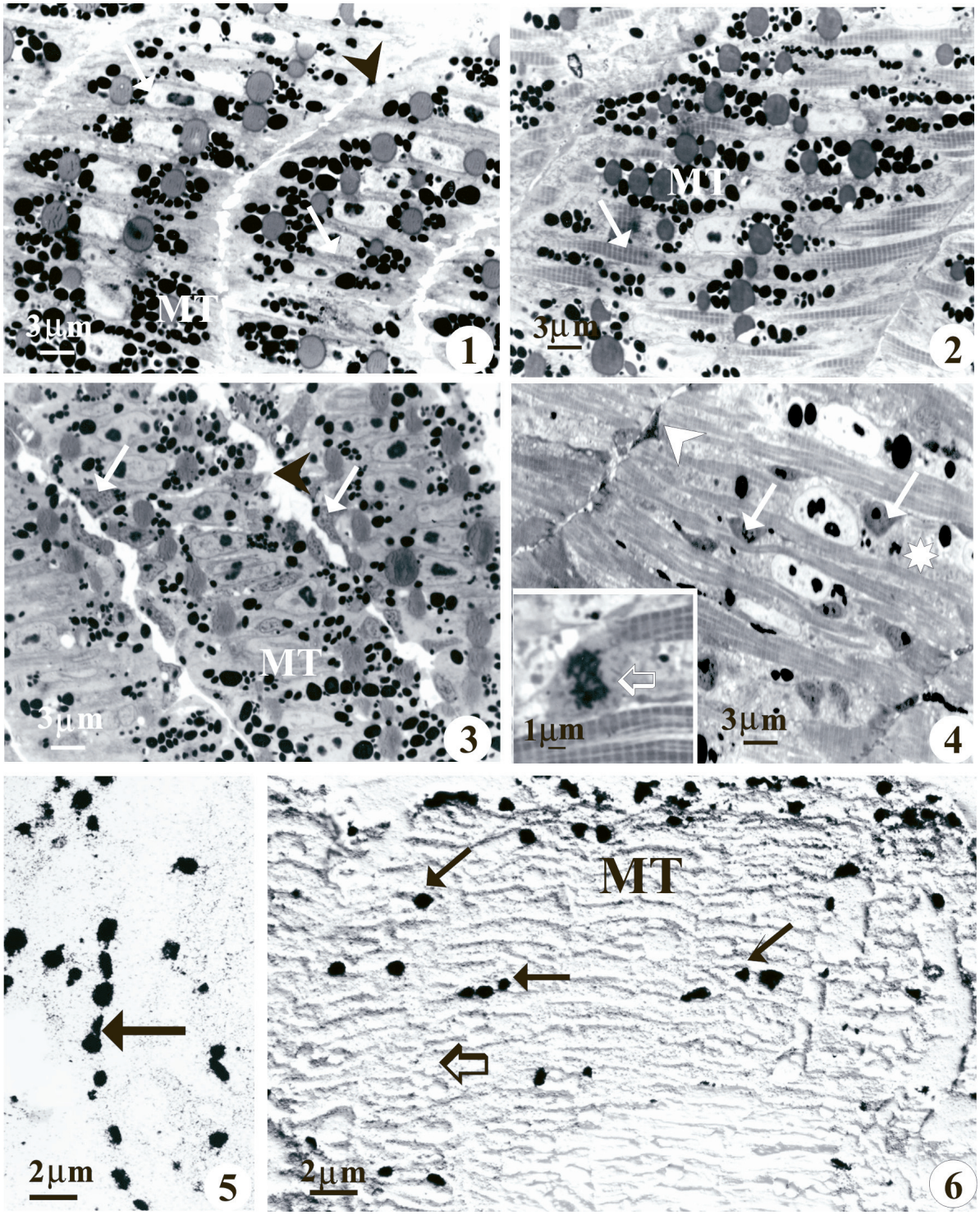

Fig. 1. Sagittal section through the embryo at stage 30. Semithin section, methylene blue. Myotome (MT), intermyotomal fissure (black arrowhead). Fig. 2. Sagittal section through the embryo at stage 35. Semithin section, methylene blue. Myotome (MT), first myofibrils (white arrow). Fig. 3. Sagittal section through the embryo at stage 37. Semithin section, methylene blue. Myotome (MT), intermyotomal fissure (black arrowhead), mesenchymal cells (white arrows). Fig. 4. Sagittal section through the embryo at stage 40. Semithin section, methylene blue. Mononucleate myotube (asterisk), mesenchymal cells in intermyotomal fissure (white arrowhead), mesenchymal cells in myotome between myotubes (white arrows). Inset – mitotic figure in mesenchymal cell between myotubes. Fig. 5. Autoradiographic incorporation of ^{3}H-thymidine into nuclei of mesenchymal cells in intermyotomal fissure (black arrow). Stage 40. Fig. 6. Autoradiographic incorporation of ^{3}H-thymidine into nuclei of mesenchymal cells (black arrows) in myotomes (MT) between myotubes (hollow arrow). Stage 40.

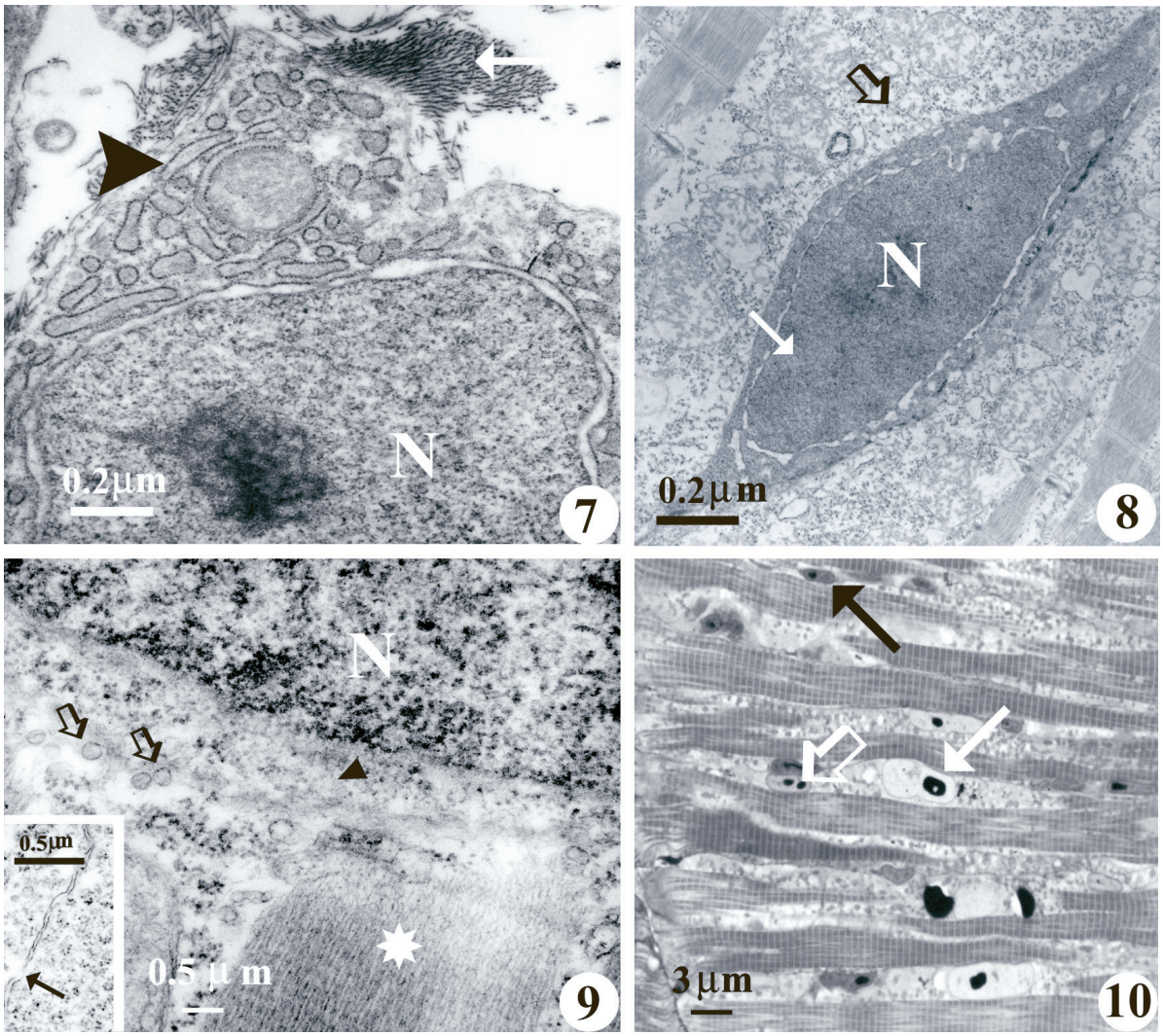

Fig. 7. Ultrastructure of mesenchymal cells in intermyotomal fissure. Nucleus (N), rough endoplasmic reticulum (black arrowhead), collagen fibres (white arrow). Stage 40. Fig. 8. Ultrastructure of mesenchymal cell in myotome between myotubes (white arrow), nucleus of mesenchymal cell (N), cytoplasm of myotube (hollow arrow). Stage 40. Fig. 9. Mesenchymal cell (secondary myoblast) (black arrowhead) adjoining myotube (white asterisk), numerous vesicles in myotube cytoplasm (hollow arrows). Inset – gaps in cell membranes between secondary myoblast and myotube. TEM. Stage 40. Fig. 10. Sagittal section through the embryo at stage 45. Semithin section, methylene blue. Large nuclei of primary myoblast (white hollow arrow), smaller nuclei of secondary myoblast (white solid arrow), mesenchymal cell (secondary myoblast) in myotome between myotubes (black arrow).

involved all myotomal cells simultaneously and, like somitogenesis, showed an antero-posterior gradient.

Mesenchymal cells located in previously empty intermyotomal fissures were first observed at stage 37. Initially these cells closely adhered to the terminal parts of the mononucleate myotubes (Fig. 3). Mesenchymal cells not adhering to the myotubes than appeared in the intermyotomal fissure**.** At later stages (38-40), the mesenchymal cells were also present in the myotomes, between the myotubes. Their cytoplasm, compared to the myotube cytoplasm, stained stronger with methylene blue (Fig. 4). Mitotic figures were observed in the mesenchymal cells occupying intermyotomal fissures and in the cells located between the myotubes (Fig. 4 inset). The pathway of mesenchymal cells was traced through the incorporation of ^{3}H-thymidine during myogenesis in *X. laevis*. Section examination revealed ^{3}H-thymidine incorporation in the nuclei of mesenchymal cells located mainly in intermyotomal fissures in younger myotomes (stage 40) (Fig. 5), and in the nuclei of these cells situated in older myotomes between the myotubes (stage 40) (Fig. 6). Nuclei of differentiating myotubes did not incorporate 3H-thymidine.

TEM analysis of the cells occupying the intermyotomal fissures (stage 40) showed that their cytoplasm contained a well-developed system of rough endoplasmic reticulum with electron dense contents. Numerous collagen fibres were observed next to fibroblasts (Fig. 7).

The cytoplasm of mesenchymal cells located between the myotubes, called secondary myoblasts,

contained numerous ribosomes and mitochondria. No collagen fibres were observed next to these cells (Fig. 8). The plasmalemmas of the myotubes and secondary myoblasts adhered closely. There were numerous vesicles close to the plasmalemma, in the myotube cytoplasm (Fig. 9). Electron microscopy demonstrated that cell membranes of adjacent myogenic cells are interrupted at particular sites (Fig. 9 inset).

At stage 45 the myotubes were first observed to contain two classes of nuclei: large nuclei of the primary myoblasts (of myotomal origin) and smaller nuclei of the secondary myoblasts (of mesenchymal origin) which had been acquired through fusion with the myotube (Fig. 10).

Discussion

The longest discussion in the history of studies concerning myogenesis of chordate skeletal muscles (about a hundred years) considered the formation of multinucleate muscle fibres. It debated whether multinucleate muscle fibres arose through mitotic and/or amitotic division of the nucleus without accompanying plasmotomy, or through fusion of cells.

The problem was finally resolved through *in vitro* studies. It was unambiguously found that the muscle fibre was a syncytium, and that myoblasts fused with one another in the G1 phase of the cell cycle. Moreover, it was found that the myotube nuclei did not replicate DNA (CAPERS 1960; KONIGSBERG *et al*. 1960; COOPER & KONIGSBERG 1961; STOCKDALE & HOLTZER 1961; KONIGSBERG 1963; STREHLER *et al*. 1963; BISHOFF & HOLTZER 1969). Model tissues for *in vitro* studies were developing pectoral muscles of chicken or quail, or chicken limb muscles. Results obtained in studies of chicken limb embryogenesis (SCHÜSSLER 1974) and a limb of *X. laevis* (KIEŁBÓWNA 1980) were similar to the ones described above. However, they pertain only to muscles of mesenchymal origin. Their characteristic common feature is asynchronous formation of myotubes (COLEMAN *et al*. 1978; BUCKLEY & KONIGSBERG 1974; ISHIKAWA 1977).

It has been previously demonstrated that amphibian myotomal myogenesis does not conform to the classic model of myogenesis of mesenchymal muscles. In myotomal myogenesis of *X. laevis*, *B. variegata* and *H. boettgeri* the myoblasts do not fuse. They differentiate synchronously in each myotome (KIEŁBÓWNA 1966; KIEŁBÓWNA & KOŚCIELSKI 1979; DACZEWSKA 2001). One of the consequences of this synchrony is a limited development potential of the myotomal cells. The myotomal cells are capable only of development into mononucelate myotubes. Their further development involves mesenchymal cells which appear in the myotomes at a later stage and fuse with the myotube (KIEŁBÓW NA & KOŚCIELSKI 1979; DACZEWSKA 2001).

Ignoring the implication of mesenchymal cells in the myotomal myogenesis of *X. laevis* resulted in different hypotheses on the origin of multinucleate muscle fibres.

In one of the first histological studies (KIEŁBÓWNA 1966), multinuclearity was presented as resulting from a smaller nucleus budding off from the large myotube nucleus with subsequent mitotic divisions of the small nucleus.

Another hypothesis was proposed by MUNTZ (1975). In her opinion satellite cells located at the ends of the myotubes fused with the myotube. Our observations indicate that initially the myotubes are not accompanied by any cells. Likewise, there are no cells in the intermyotomal fissures, and the cells adhering to the myotubes and appearing at a later stage of myogenesis are mesenchymal cells. According to MAURO (1961), the discoverer of satellite cells, these cells are dormant myoblasts located under the basal membrane of a mature muscle fibre. The cells observed by MUNTZ (1975) were most probably mesenchymal cells.

According to the third hypothesis, the multinucleate muscle fibres in the myotomes of *X. laevis* are a product of amitotic divisions of the large myotube nucleus (BOUDJELIDA & MUNTZ 1987). This suggestion is based on TEM studies. According to these authors the deeply invaginating nuclear envelope is a manifestation of the amitotic division of the nucleus. This, however, seems unlikely, since no ^{3}H-thymidine incorporation was observed in nuclei (BOUDJELIDA & MUNTZ 1987), nor DNA replication in the nuclei of mononucleate myotubes, in our studies. Thus, *in vitro* studies have demonstrated that the invaginating nuclear envelope, even reaching as far as half the diameter of the nucleus, is not due to amitotic division but rather is a morphological symptom of nucleus movement (STOCKDALE & HOLTZER 1961; COOPER & KONIGSBERG 1961).

The presented data suggest a new hypothesis and indicate a high similarity between the myotomal myogenesis of *X. laevis* and that of *B. variegata* (KIEŁBÓWNA & KOŚCIELSKI 1979) and *H. boettgerii* (DACZEWSKA 2001). Autoradiography with ^{3}H-thymidine and cytophotometric examination of nuclear DNA showed that the nuclei of mononucleate myotubes of *B. variegata* did not replicate DNA, and that their DNA quantity was tetraploid. In the present study marked nuclei were not found in the myotubes. As in *B. variegata,* in *X. laevis* the ^{3}H-thymidine-marked cells were found to migrate into the intermyotomal fissures, and then into the myotomes between the myotubes.

TEM examination revealed that the mesenchymal cells located in the intermyotomal fissures in *X. laevis,* like in *H. boettgerii* (DACZEWSKA 2001), differentiated into fibroblasts whereas the cells in direct contact with the myotube acquired myogenic capacity, perhaps under the influence of the myotube. Their myoblastic properties are confirmed by their ability to fuse with the myotube. Direct evidence for the fusion of the secondary myoblast with the myotube in *X laevis* is the presence of subsarcolemmal vesicles in the myotube cytoplasm.

These results would confirm the hypothesis of ORLOV *et al*. (1989) rather than that of KALDERON & GILULA (1979). According to ORLOV *et al.* (1989) subsarcolemmal vesicles appear in one of the fusing cells, and according to KALDERON & GILULA (1979) – in both fusing myoblasts.

Following the fusion of the secondary myoblasts with the myotube in *X. laevis,* as in *B. variegata* and *H. boettgerii* (KIEŁBÓWNA & KOŚCIELSKI 1979; DACZEWSKA 2001), the myotube nuclei show a clear dimorphism. The myotube contains a large nucleus and smaller nuclei of the secondary myoblasts, located below the sarcolemma. In the myotomal myogenesis of *B. variegata* smaller nuclei contained a diploid DNA quantity (KIEŁBÓWNA & KOŚCIELSKI 1979). The same DNA quantity was found in the small nuclei of *X. laevis* in an earlier study (KIEŁBÓWNA 1966).

Taken together, the present results indicate that in the first step of myogenesis all the myotomal myoblasts in *X. laevis* differentiate synchronously into monucleate myotubes. The second step involves secondary myoblasts of mesenchymal origin that migrate into the myotomes and fuse with primary myotubes to form syncytial muscle fibres.

The closely related *X. laevis* and *H. boettgeri* (Pipidae), and *B. variegata* (Discoglossidae) conform to the same model of myotomal myogenesis.

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