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Review

Developmental histories in amphibian myogenesis

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I would on first setting out, inform the reader that there is a much greater number of miracles and natural secrets in the frog than anyone hath ever before thought of or discovered.

Jan Swammerdam, 1758

Introduction

Muscle development has captured the attention and imagination of generations of biologists. Much of the fascination comes from the intriguing structural and functional diversity of mature muscle fiber types. Diversity occurs, however, in all aspects of muscle development, including establishment of muscle precursors, patterns of gene expression, mechanics of tissue morphogenesis, and modulation of fiber type through use, innervation, or hormone levels. Underlying this diversity, however, is a common theme, referred to in this review as developmental history. By developmental history, we mean that at various stages in muscle cell development, critical historic events occur that limit or constrain successive stages of muscle cell development. As in human history, the outcome of each developmental event depends on preceding history, prevailing conditions, and, to some extent, chance. This view of development contrasts with the notion that development is the expression of a rigid, predetermined program that unfolds as a predictable, inexorable series of events set in motion at fertilization. Our view is that development has been patched together through evolution in a way that is often elegant, but not necessarily logical, predictable, or efficient. Because they are unpredictable, developmental processes are best understood when viewed retrospectively, backwards from the final phenotype, rather than looking forward from the egg or early embryo.

This review focuses on developmental histories during amphibian myogenesis. Amphibians are valuable for studying myogenesis for several reasons. First, they present several technical advantages. (1) Early embryological stages are easily accessible, so that early cleavage, blastula, and gastrula stages, when mesoderm is specified and muscle cell lineages are established, can be conveniently observed. (2) Embryonic cells are readily cultured. Their internal yolk stores permit them to survive and differentiate in simple, defined salt solutions. (3) Eggs and embryos can be manipulated surgically. (4) Exogenous molecules can be injected into egg cells or embryos. For example, cell lineage markers, foreign DNA, antibodies, and growth factors have all been successfuly introduced into amphibian embryos.

Second, in addition to these experimental advantages, amphibian myogenesis offers many conceptual advantages. (1) The rich annals of amphibian embryology provide an unequaled background for current molecular research in determination, induction, and cell lineage specification. (2) Metamorphosis provides the premier model for thyroid hormone regulation of muscle remodeling.(3) Amphibian muscles share features of both amniote and lower chordates, and so are important for comparative studies of myogenesis.

This review integrates current information about amphibian mesoderm specification, the origin and location of muscle cell precursors, possible myogenic lineages, and morphogenetic movements of muscle precursors and myoblasts. It discusses the number and identity of amphibian fiber types, molecular events during differentiation, the role of hormones and remodeling during metamorphosis and regeneration, and possible evolutionary relationships between myogenesis in amphibians and other organisms. Because the net is cast wide, it is not possible to include all the relevant details. Instead, we have extracted the key questions: those that are currently being intensely investigated, and those that need to be investigated soon.

Mesoderm specification and the establishment of muscle cell lineages

Myoblast precursor cells arise from mesoderm. Full understanding of myogenesis, therefore, requires an

Abbreviations used in this paper: myHC, myosin heavy chain; FGF, fibroblast growth factor; TGF, transforming growth factor; PMM, primary myotome myofiber; N-CAM, neural cell adhesion molecule.

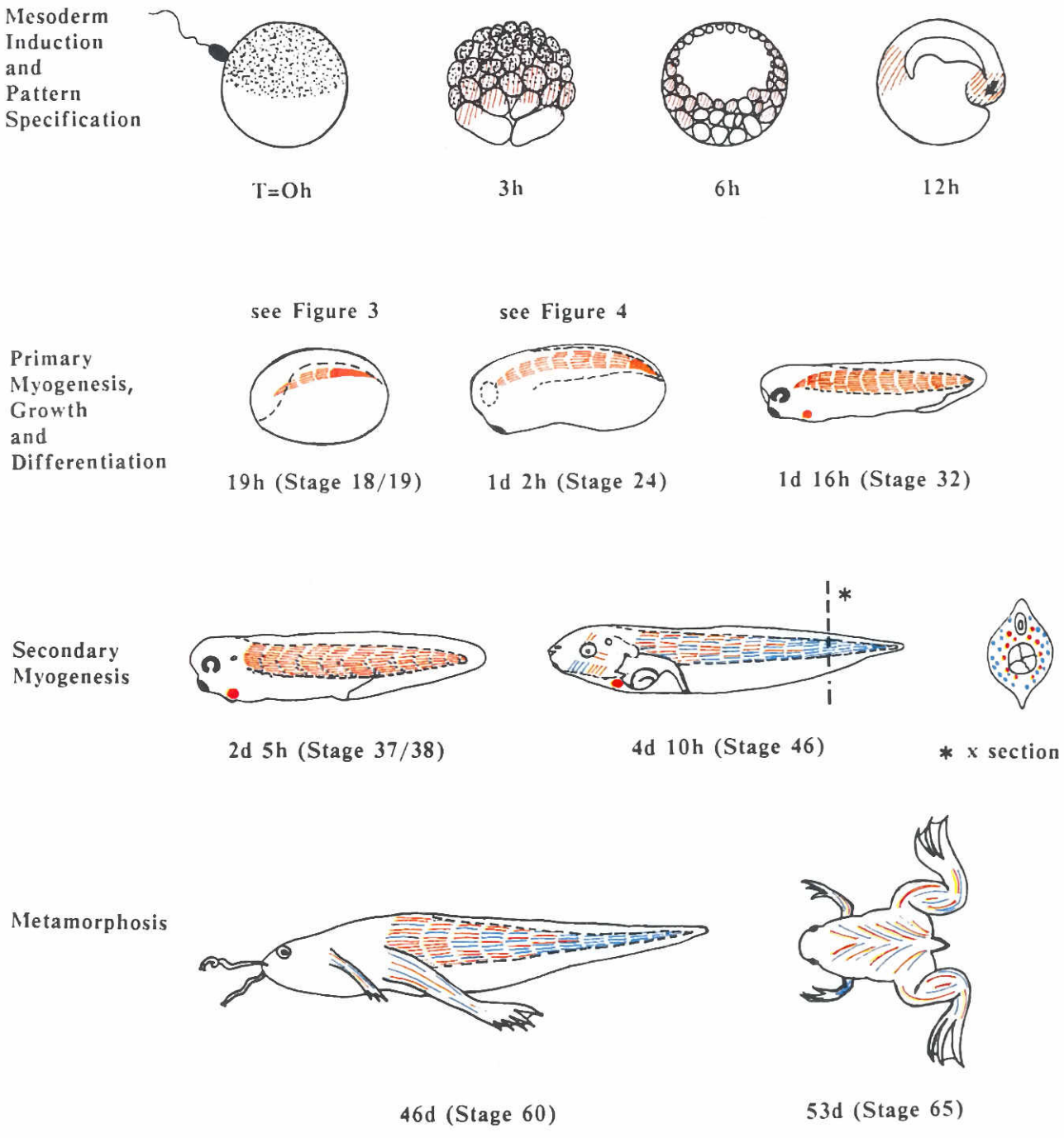

Fig.1. Myogenesis in the anuran *Xenopus laevis*. *This scheme applies generally to other amphibians that undergo metamorphosis. However, the timing and details of myogenic events may differ substantially among species (see Fig. 2). Developmental stages are divided arbitrarily into three groups. The first (early events) emphasizes mesoderm induction. During cleavage stages cells from the equatorial region (brown) are induced to become mesoderm, probably by a signal emanating from vegetal cells. Muscle cells develop from part of this population. The second group emphasizes initial differentiation and growth of muscle cells. Unsegmented paraxial mesoderm, axial myoblasts, primary axial myocytes, and heart (all in red) derive from mesoderm induced at earlier stages. The third phase includes remodeling of the primary myotomal muscles. At stage 36, mesenchyme cells (blue) migrate into intermyotomal septa and may give rise to secondary myoblasts. At stage 46, fast (red) and slow (green) fibers are detected. Finally, muscles are remodeled again during metamorphosis. In adults, up to three fast-fiber types and two or three slow-fiber types can be detected (see Table 1); for clarity only two are shown. Stages and developmental times are taken from Nieuwkoop and Faber (1975).*

examination of mesoderm induction. A complete discussion of this complex topic is beyond the scope of this review (see Dawid *et al.*, 1989 and Smith, 1989 for excellent recent summaries). Instead, we concentrate on questions directly related to myogenesis.

When do the cells of the early embryo become committed to form mesoderm?

There is no single event that determines amphibian mesoderm. Instead, prospective mesoderm cells begin to be specified (reversibly committed) shortly after fertilization, when the radial symmetry of the egg is broken and the dorsal/ventral axis is established. Specification is not complete, however, until around the sixth cleavage (64-cell stage). This simple answer is based upon the following observations. Explants of marginal zone cells (Fig. 1) prepared prior to the 64-cell stage fail both to express muscle-specific genes such α-actin (Gurdon *et al.*, 1985a), and to display cytological features that characterize mesoderm derivatives (Nakamura and Matsuzawa, 1967; Nakamura *et al.*, 1970). Marginal zone cells explanted after this stage, however, yield the full spectrum of mesoderm derivatives, including notochord, muscle, pronephros, and blood cells (Nakamura and Takasaki, 1970).

Is mesoderm specified by cell-cell interactions or by signals localized in the egg during oogenesis?

Embryological manipulations strongly support the view that vegetal hemisphere blastomeres induce equatorial zone cells to become mesoderm. In the most informative experimental analyses, specific zones of the early blastula animal cap were cultured alone or with blocks of vegetal hemisphere cells (Ogi, 1967, 1969; Nieuwkoop, 1969; Sudarwati and Nieuwkoop, 1971). Since only those recombinants that contained both animal and vegetal hemisphere cells produced mesoderm derivatives, and those derivatives came from animal cap cells and not from vegetal cells, it was concluded that "the mesoderm develops exclusively from the ectodermal half of the egg under the influence of an inductive action from the part of the endodermal half" (Nieuwkoop, 1969).

More recently, composite embryos prepared from various combinations of 8-cell stage *Xenopus* blastomeres yielded the same conclusion (Kageura and Yamana, 1986), as did manipulations in which actin mRNA accumulation was monitored rather than cytological characteristics (Gurdon *et al.*, 1985b). Furthermore, Boterenbrood and Nieuwkoop (1973) demonstrated that dorsal side blastomeres from the vegetal hemisphere induce notochord and muscle. Conversely, ventral blastomeres of vegetal hemisphere origin induce primarily blood islands. Thus, the inducing capacity of vegetal hemisphere blastomeres is regionally specified along the dorsal/ventral axis.

Blastomere transfer experiments have recently provided data consistent with the above findings. Mild ultraviolet irradiation of the vegetal hemisphere of the fertilized egg generates defects in embryonic dorsal structures (Malacinski *et al.*, 1977). When vegetal hemisphere blastomeres from the dorsal side of irradiated embryos are surgically replaced with equivalent blastomeres from control embryos, the irradiated embryos are rescued (Gimlich and Gerhart, 1984).

Although the experiments just described provide a coherent picture of mesoderm induction, several criticisms can be offered that advise caution about accepting the results wholesale. First, although vegetal blastomeres can induce animal cap cells to differentiate into mesoderm derivatives in culture, this does not necessarily mean that in normal embryogenesis the mechanism for mesoderm formation is the same. Also, it should be recalled that lithium ion can similarly cause cultured animal cap cells to differentiate into mesoderm (Nieuwkoop, 1970).

Second, the bioassay, in which groups of cells are cultured and observed several days later for cytological differentiation, is inherently complex and severely constrained. Four shortcomings need to be seriously considered. First, negative data are heavily weighted. That is, failure of cells to differentiate is scored to mean that cells are uncommitted, or not yet programmed to differentiate. A strong positive statement is therefore based on negative data. Second, the end point assay – histological differentiation – no doubt requires a long chain of steps. Should any one of them fail, a negative result at the endpoint will be recorded. Third, the bioassays are often done with animal pole cells as responding tissue, while in the embryo it is equatorial cells that become mesoderm. Finally, phylogenetic differences between test species are often ignored. Both the anuran *Xenopus* and various urodeles such as *Ambystoma* were tested for the capacity of vegetal hemisphere blastomeres to induce overlying ectoderm cells to develop into mesoderm. The results were the same in both groups, suggesting that at least in broad outline a similar mechanism may be common among all amphibia. However, *Xenopus* mesoderm arises internally from blastomeres that contact directly vegetal hemisphere cells, whereas much of the urodele mesoderm arises from animal hemisphere cells that reside on the surface of the embryo (Smith and Malacinski, 1983). Because they are separated from vegetal blastomeres by a long distance, urodele prospective mesoderm cells are unlikely to receive a signal directly from the vegetal hemisphere, as is the case for *Xenopus*. Although direct contact between inducing and responding cells may be required for mesoderm induction in *Xenopus* (Sargent *et al.*, 1986), the signal must diffuse or be serially propagated to reach the distantly located prospective mesoderm cells on the urodele embryo's surface. To date, this aspect of mesoderm formation has often been

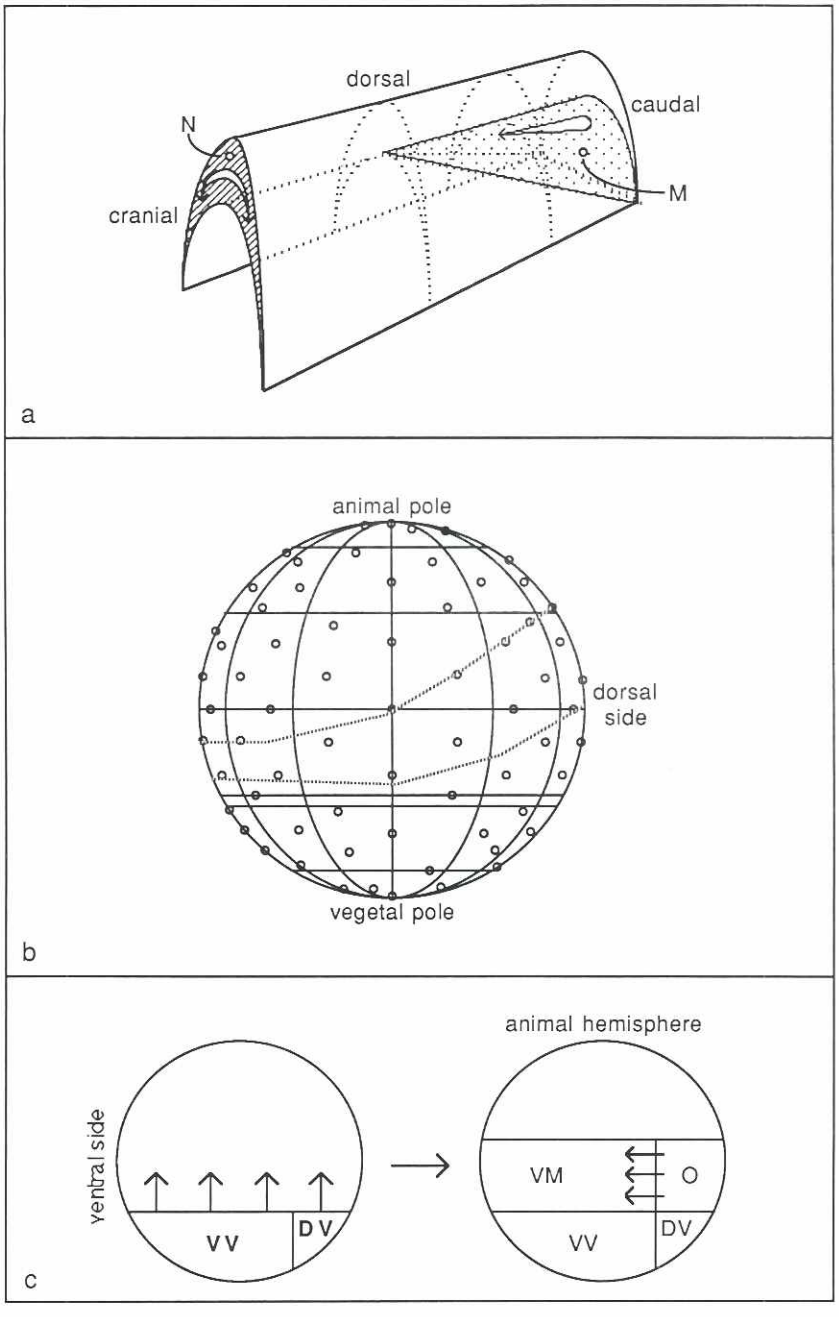

Fig. 2. Examples of models proposed to explain mesoderm induction in amphibian embryos. (a) *The theory proposed by Toivonen and Saxén (1955) deploys two gradients on the dorsal side of the embryo. A neuralizing principle (N) is strongest in the dorsal midline and decreases in a gradient laterally. A mesodermalizing principle (M) is at its highest concentration along the caudal midline. It decreases in intensity, as a gradient, both cranially and laterally. During embryogenesis the N and M components interact to generate local inductions. Where the M principle is at its highest concentration (caudally), trunk and tail muscle is induced.* **(b)** *Mesoderm induction is described by Weyer* et al. *(1977) as the result of a diffusion-reaction process on the surface of a spherical embryo (blastula). A morphogen is continuously produced by the endoderm, and diffuses so that the concentration gradient is stable. Points reflect the concentration of the morphogen. The contoured lines circumscribe an isoconcentration zone where mesodermal derivatives are induced.* **(c)** *The three-signal model proposed by Dale and Slack (1987) envisions two mesoderm induction signals emanating from the vegetal hemisphere. A dorsal/vegetal signal (DV) induces dorsal mesoderm (including the primary embryonic organizer [O]) and a ventral/vegetal (VV) signal induces ventral mesoderm (VM). The organizer region then induces the ventral mesoderm to form more muscle and pronephros. Each of the above illustrations was redrawn from the references cited above.*

neglected in discussion of models of the type discussed below. Perhaps it should be recalled that not only does the mesoderm's spatial origin differ among phylogenetically distinct species, but the origin of primordial germ cells is likewise very different in the two most popular test species, *Xenopus* and *Ambystoma* (Sutasurja and Nieuwkoop, 1974). The phylogenetic distance between these two major groups of amphibians and their distinct differences in known embryogenic pathways caution us about extrapolating to all amphibia the results gained from studying *Xenopus* or any other single species. The evolutionary histories of different amphibian species may have created divergent developmental histories.

What models have been proposed to account for mesoderm induction?

Fig. 2 illustrates three representative models, all of which should be considered "holistic" in that they attempt to integrate each experimental model into a coherent view of how mesoderm induction occurs.

"Heuristic" models, which are designed more to discover how a process works, would, however, be more useful. The holistic models fail to generate experimental tests that go beyond the obvious embryological manipulations, most of which have already been pushed to their limits (see critique below). Unfortunately, heuristic models were difficult to formulate until the recent discovery that various growth factors probably participate in the induction of mesoderm (see below). Thus, exciting new advances should now lead to a new generation of models with more intellectually satisfying molecular features.

What is the biochemical basis of mesoderm induction?

Recent evidence suggests that peptide growth factors are the endogenous mesoderm inducers.

Beginning with Toivonen's 1953 discovery that cultured urodele ectoderm could be induced to differentiate into mesodermal derivatives if bathed in an extract of guinea pig bone marrow, a wide variety of crude extracts or partially purified preparations from heterologous sources have been demonstrated to induce mesoderm (recently reviewed by Dawid *et al.*, 1989; Smith, 1989).

More recently, a major advance in understanding mesoderm induction occurred when Smith (1987) reported that supernatants from a *Xenopus* XTC cell line contain a mesoderm inducer. Subsequently, the active molecule was found to be similar to transforming growth factor peptide TGF-β2, which had identical activity in the usual animal pole explant culture bioassay (Rosa *et al.*, 1988; Smith *et al.*, 1988). Since the initial report, other growth factors, most notably fibroblast growth factor (FGF), have been tested and have provided positive results. Each of these growth factors acts differently in the bioassay. According to Smith *et al.* (1988), TGF-β2 induces the development of a broad range of mesoderm derivatives, including muscle, notochord, kidney, mesenchyme, and mesothelium. Basic -FGF, however, although it induces most of those mesoderm derivatives, fails to induce notochord (Godsave *et al.*,1988). More recent experiments indicate that basic FGF translated *in vitro* from a bovine cDNA is more active than the protein extracted from bovine brain and does induce notochord in the bioassay (Paterno *et al.*, 1989). The basis for this increased activity is not known.

Not only have these purified, heterologous growth factors tested positive in bioassays, but direct evidence has also been obtained for the presence of homologues in *Xenopus* embryos. An FGF mRNA has been found in *Xenopus* oocytes, eggs, and embryos, and its protein product is present in concentrations adequate for mesoderm induction (Kimelman *et al.*,1988; Slack and Isaacs, 1989). A TGF-β like mRNA, known as Vg1, has also been identified and found to be localized in the vegetal hemisphere of *Xenopus* eggs (Melton, 1987). Its protein product is translated only in the vegetal hemisphere of oocytes; then it diffuses into the animal hemisphere, probably through the endoplasmic reticulum, so that by the time of fertilization it is present in both animal and vegetal hemispheres (Dale *et al.*,1989).

These studies suggest, therefore, that more than one peptide growth factor contributes to mesoderm pattern specification, and furthermore, that these growth factors may act in combinations, as predicted by some gradient models (Ruiz I Altaba and Melton, 1989a, b). In addition, it is known that under some experimental circumstances, (e.g. cell cultures of rat and chicken embryo myoblasts) TGF-β1 and FGF *inhibit* myogenic differentiation. The inhibition is reversible upon removal of TGF-β1 from the medium (Massague *et al.*, 1986; Spizz *et al.*, 1987). *Xenopus* XTC-MIF inhibits the expression of epidermal cell proteins in animal cap cultures (Smith *et al.*, 1988). Thus, there may be families of endogenous growth factors whose members may have opposite effects on myogenesis.

Does mesoderm induction create a rigid lineage of muscle cell precursors?

Overwhelming evidence indicates that early amphibian embryos, generally considered to be "regulative" rather than "determinative", do not create a uniform muscle cell lineage. This conclusion was anticipated by the observation that early embryonic cleavage patterns vary substantially among different batches of eggs. The third cleavage furrow of *Xenopus* eggs, for example, occasionally forms vertically rather than horizontally, generating a rosette. These eggs often develop perfectly normally. Hence, a highly regulative early embryo such as *Xenopus* is unlikely to yield a predictable cell lineage map of the type observed in highly determinative embryos such as *Caenorhabditis elegans* (Sulston *et al.*, 1983). Various tracers have been used to follow cell lineages in amphibian embryos, and these studies confirm that progeny of a given blastomere can contribute to all tissues of later stage embryos, and the distribution varies from embryo to embryo (Dale and Slack, 1987; Moody, 1987; Jacobson and Xu, 1989).

If strict muscle lineages are established, this must occur rather late in development, perhaps at gastrulation. Embryological information suggests that, at a minimum, the cardiac and skeletal muscle cells represent distinct populations. In *Xenopus*, for example, cardiac and skeletal muscle cells arise from different regions of the embryo at different times during early development. As already discussed, skeletal muscle cells are specified before gastrulation and begin to differentiate in axial mesoderm by the end of gastrulation. In contrast, cardiac muscle is induced during gastrulation in *Xenopus* (Sater and Jacobson, 1989) and midneurulation in *Ambystoma* and develops from lateral

mesoderm after the tailbud stage (Jacobson and Duncan, 1968; Lemanski *et al.*,1979). Also, mesoderm induced in cultured animal hemisphere cells by peptide growth factors, as described earlier, apparently produces skeletal, not cardiac muscle. Together, these results indicate that muscle cell lineages, should they exist, are established late in development, and not at early cleavage stages.

Prospects and perspectives

Since peptide growth factors are now prime candidates for the endogeneous mesoderm inducers, half of the difficult task of understanding mesoderm induction appears to have been accomplished. That is, well-defined, fully characterized effector molecules have been identified and are commercially available for routine experimentation. The remaining half of the task is to understand their mechanism of action. Although seemingly complex, this project should progress relatively quickly, considering the large number of laboratories involved in the research. The following critical questions remain.

(1) Are mesoderm-inducing peptide growth factors localized in the vegetal blastomeres, as would be predicted from embryological experiments? Are they present in concentration gradients? Specific antibodies are needed to establish their exact location within the embryo.

(2) Can mesoderm be induced in the absence of peptide growth factors? Lacking a practical developmental genetics strategy, this question can perhaps be answered by deleting FGF and Vg1 from early embryos, by attacking the message with antisense nucleic acids, or by defeating the protein itself with analogs or antibodies injected into embryos. Despite valiant attempts, to date none of these approaches has succeeded. If several growth factors act in combination or in opposition during mesoderm induction, progress will be exceedingly slow.

(3) What is the molecular mechanism of growth factor activity? It should be remembered that the equatorial zone cells become committed to form mesoderm by the 6th cleavage, whereas the embryo does not begin transcription until the midblastula transition, at the 10-11th cleavage. Do factors activate pre-existing components of equatorial zone cells? Do they act first by binding to surface receptors as do other growth factors, or do they or their secondary messengers invade the responding cell's nucleus, remain dormant until the mid-blastula transition, then activate specific gene expression patterns?

(4) Do the topological distribution and mechanism of action of peptide growth factors explain the holistic models, which account for the majority of the embryological data? The Vg1 mRNA, for example, is localized primarily in the vegetal hemisphere (Melton, 1987). During the time of its presumed activity its protein product can be immunoprecipitated from equatorial cells as well as vegetal cells. Whether it is present as a gradient, as some of the models predict, is not known (Dale *et al.*, 1989).

(5) What other growth factors and developmentally important gene products regulate mesoderm formation? For example, there is recent evidence that the cellular oncogenes INT-2 and kFGF may be involved (Paterno *et al.*, 1989). Moreover, regional specification within the mesoderm may be linked to graded expression of homeobox genes (Ruiz I Altaba and Melton, 1989a, b).

(6) What is the molecular mechanism of competence? A cell's competence to respond to induction signals can be assayed at the embryological level (e.g., Chung and Malacinski, 1983), but that approach is subject to most of the limitations mentioned previously for the induction bioassays. A more productive approach probably would be to search for growth factor receptor molecules. A start on this problem has been made by Gillespie and co-workers (1989), who have found that the density of cell surface receptors for FGF precisely follows the time course for competence of the animal pole explant's response to FGF. However, on the surfaces of vegetal cells they also found receptors which do not make mesoderm derivatives in response to FGF. They also could detect no gradient of FGF receptors along either the animal/vegetal or dorsal/ventral axis. Thus, the mere appearance of receptors for growth factors, while necessary, is not sufficient to explain competence.

(7) To what extent will results obtained with *Xenopus* apply to other anurans and to urodeles? Since even lithium ion has mesodermalizing activity, it cannot be stated for certain that FGF/TGF-β are universal amphibian mesoderm inducers. However, it is possible that lithium ion merely potentiates a second messenger part of the induction cascade (Cooke and Smith, 1988; Slack *et al.*, 1988). If so, other molecules, not just peptide growth factors, could conceivably elicit the appearance of the second messenger in some species and hence induce mesoderm. There is ample evidence for the existence of redundant pathways for important developmental programs (Malacinski and Neff, 1989). Perhaps there are redundant or alternate mechanisms in embryos that can substitute for FGF or Vg1 by eliciting the same second messenger pathway. Each species could use a unique combination of growth factors to initiate similar developmental programs.

(8) What molecular mechanism determines muscle cell lineage? This key question has no answer yet. However, in a spate of recent studies on amniote cells,

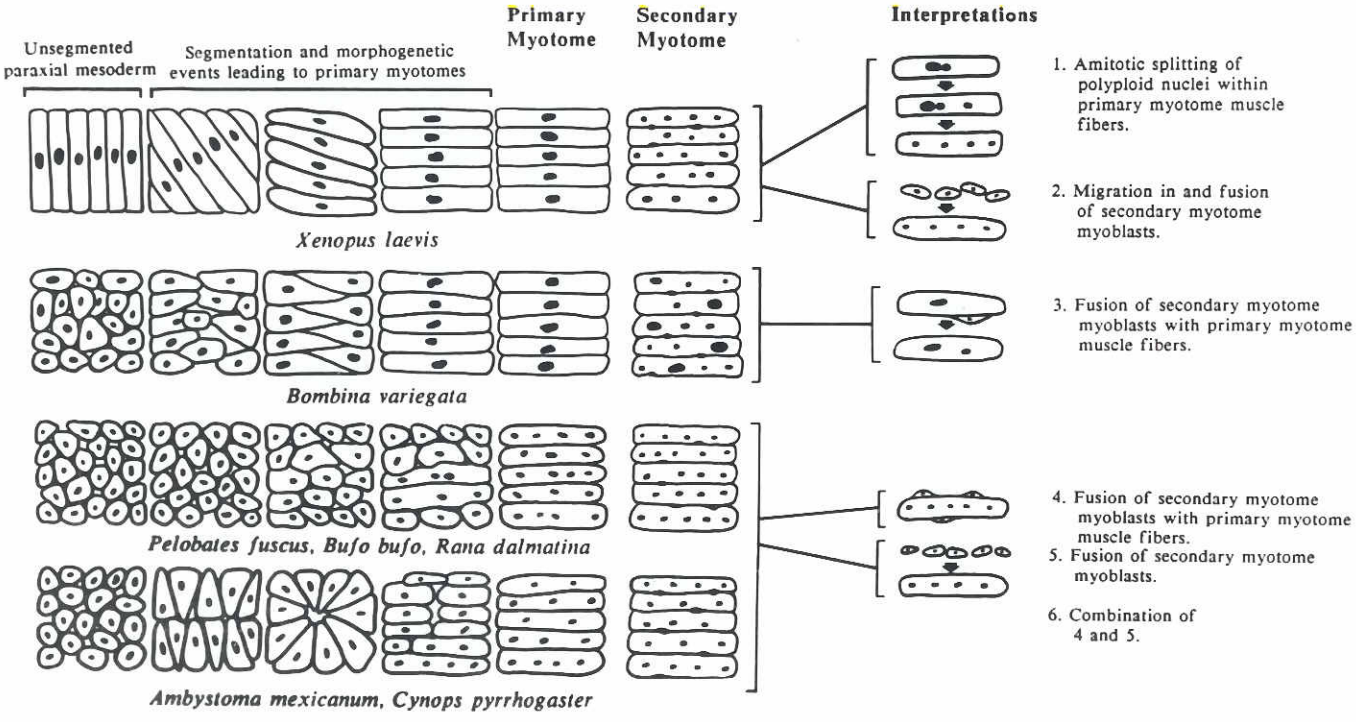

Fig.3. Diverse pathways for segmentation and differentiation of myotomes in amphibia. *From left to right are successive stages of myotome development in several species. Primary myotomes in some species comprise a pure population of mononucleated myocytes, and in other species multinucleated myotubes. Secondary myotomes contain interstitial cells, some of which are satellite cells. Nuclei of primary multinucleated myotubes are diploid. However, primary myocytes in* X. laevis *and* B. variegata *are polyploid (Kielbowna, 1966; Kielbowna and Koscielski, 1979). Secondary myotome fibers can apparently arise by fusion of myotome myoblasts, or by other morphogenetic pathways including amitosis (Boudjelida and Muntz, 1987) and fusion of diploid and tetraploid myoblasts with primary myotome fibers (Kielbowna and Koscielski, 1979).*

several genes have been characterized that appear to regulate determination of the myogenic lineage. The genes myoD1 (Tapscott *et al.*, 1988), myd (Pinney *et al.*, 1988), and myogenin (Edmondson and Olson, 1989; Wright *et al.*, 1989) constitute a family of related genes that can convert transfected fibroblast cells into skeletal muscle cells. They probably encode transcription factors that are part of a regulatory cascade for determination. It seems likely that families of homologous genes will be found in amphibia. If so, it will be of great interest to learn whether mesoderm-inducing factors activate any of these genes, experiments that should be easy to perform with the cultured animal cap assay.

To summarize, the picture arising from such disparate sources as classical and comparative embryology, cell biology, molecular biology, and growth factor studies suggests the following speculative model for muscle cell specification. It begins with a growth factor signal initially localized in the vegetal hemisphere. This growth factor diffuses into the equatorial zone before or during early cleavage stages, where it either binds to receptors on the surface of equatorial cells or acts internally. The signal is transduced by a second messenger system that is sensitive to lithium ion, such as the phosphoinositol cascade (Busa and Gimlich, 1989). Over the next several cleavages the second messenger causes the expression or activation of a series of myogenic determination genes. These genes produce transacting transcription factors during and after gastrulation that initiate the expression of sarcomeric gene transcripts, while maintaining their own expression, thereby stabilizing determination.

Developmental programs of different muscle groups

The events discussed above are largely complete by gastrulation. However, at this stage only a small portion of the developmental history of muscle cells has been accomplished. Amphibians change drastically both in form and function during their life cycle. Embryos, larvae, tadpoles and adults have distinct and different life-styles and hence require unique muscle functions appropriate for each stage. These different needs are reflected in the number of different programs for muscle development (Fig. 1). Moreover, between amphibian orders, and even between species, muscles form in strikingly diverse ways. These variations provide valuable material for comparative studies of myogenesis.

This section reviews the cellular origins of muscle diversity and mechanics of muscle histogenesis.

What morphogenetic mechanisms produce primary myotome?

In all amphibia studied thus far, the paraxial mesoderm segments into somitomeres, each of which gives rise to a somite (Fig. 3; see Jacobson, 1988 for a review). Each individual somite then divides into a centrally located myotome, a dorsolateral dermatome and a ventromedial mesenchymal sclerotome. The myotome predominates, the dermatome is relatively small, and the sclerotome limited to only a few cells (see Malacinski *et al.*, 1989, for review). These first, or primary, myotomes comprise a pure population of differentiating myotubes. There are no interstitial cells in the primary myotome (Kielbowna, 1966).

Although probably all amphibian myotomes share these early characteristics, further cellular and morphogenetic processes leading to functional myotomal muscle differ surprisingly between species (Fig. 3). For example, in *Xenopus laevis*, primary myotome myofibers (PMM) are mononucleated, but, interestingly, the nuclei become polyploid up to octaploid (Kielbowna, 1966). In *Bombina variegata*, PMM fibers are mononucleated and have a tetraploid amount of DNA per nucleus (Kielbowna and Koscielski, 1979). In *Bufo bufo, Rana dalmatina*, and *Pelobates fuscus* , PMM fibers are multinucleated, apparently with a diploid amount of DNA per nucleus (Brustis, 1979; Kielbowna and Koscielski, 1981). Urodele PMM fibers studied thus far are multinucleated (Loeffler, 1969; Youn and Malacinski, 1981a, b). The DNA content of these nuclei has not been investigated.

Cellular rearrangements within the myotome also vary widely (Fig. 3). In *Xenopus*, myoblasts initially orient perpendicular to the axis of the unsegmented paraxial mesoderm, then rotate through 90 degrees to lie parallel to the axis with each PMM fiber spanning the length of a single myotome (Hamilton, 1969; Youn and Malacinski, 1981a). Other modes of PMM myotomogenesis have been reported. In *Bombina*, the newly segmented PMM sort out from each other, whereas *Pelobates* myotome fibers form by fusion of PMM myoblasts (Kielbowna, and Koscielski, 1981). Thus, although the resulting myotome architectures are similar, they are achieved by strikingly different pathways. This observation further compels us to abandon the idea that there is a single, universal program for myogenesis, for it seems unlikely that such a program could be so easily modified during evolution.

What are the different skeletal muscle fates?

An amphibian skeletal muscle can have one of at least five destinies, at least three of which result in cell death before adulthood. The first fate, early embryonic death, occurs in the cranial-most myotomal muscles. Fibers from these myotomes (cranial myotomes W, X, Y, and Z, trunk myotome S1 and one half of S2 in *Xenopus*), are the first in the embryo to differentiate, and in *Xenopus* they even become innervated and functional (Ryke, 1938; Smit, 1953; Regel and Epstein, 1972; Blackshaw and Warner, 1976). However, they also undergo autonomous, programmed cell death in a cranial/caudal sequence beginning at about stage 26 and ending by stage 52 (Ryke, 1938; Smit, 1953; Chung *et al.*,1989). Trunk myotome muscles display the second fate, persistence through adulthood. Most survive metamorphosis and persist in adults, including those that become the *latissimus dorsi* (Ryke, 1938). The third fate, late embryonic death, is met by tail myotome fibers, which differentiate during larval stages, then degenerate during metamorphosis. The fourth fate, replacement, is described by the adductor jaw muscles, which differentiate during early embryogenesis but die and are replaced by adult myocytes during metamorphosis (Alley, 1989). Finally, a fifth fate, late development, is displayed by limb muscles, which do not appear until the middle of metamorphosis, but then remain in adult leg.

Although this scheme describes the apparent fate of muscle masses, in most cases the fates of individual myofibers within those muscles is unknown. For a given fiber there are three possible histories. It could undergo programmed cell death, persist unchanged, or alter its type by changing its pattern of gene expression. The trunk myotomal muscles in anurans are a useful example (Figs. 2 and 3). Initially they consist of functional, mononucleate fibers. Shortly after the heart beat begins, mesenchymal cells migrate into the myotomes through the intermyotomal septa (Ryke, 1938; Muntz, 1975; Brustis and Delbos, 1976; Kielbowna and Koscielski, 1979; Boudjelida and Muntz, 1987). The origin of the mesenchymal cells is not clear; they may come from sclerotome (Ryke, 1938) or dermatome (Glucksmann, 1934). After the migration, satellite cells, which presumably derive from the invading mesenchyme, appear in the myotome, and secondary, multinucleated myofibers also appear. The fate of the primary myotomal myofibers, and the origin of these secondary myotomal fibers is controversial (Muntz, 1975; Kielbowna, 1980). The primary fibers may die, as they do in the cranial myotomes. Such death would not be readily apparent in the trunk because of the migration and proliferation of secondary myoblasts. In this scenario secondary, multinucleated myofibers arise from fusion of recently migrated secondary myoblasts. Alternately, primary fibers may not die, but instead fuse with each other, or with secondary myoblasts. In this scheme, trunk primary myofibers are rescued from the programmed death fate of their cranial neighbors. Finally, it has been suggested that multinucleated secondary myotomal fibers in *Xenopus* arise from amitotic division of the

primary nuclei, hence without cell fusion (Kielbowna, 1966; Boudjelida and Muntz, 1987).

Prospects and perspectives

The picture that emerges indicates that although all skeletal muscle cells derive from mesoderm, their fates differ according to the region of the embyro in which they differentiate. Cranial, trunk, and tail myotomal muscles follow three different fates, even though they form an apparently continuous series from head to tail. The first issue is, what mechanism could account for this regionalizaton of fate? Are fates autonomous, programmed into specific lineages during early mesoderm development? Or are those fates epigenetic, dependent on, for example presence or absence of growth factors or signals provided by adjacent cells? One exciting possibility is that those fates, whether autonomous or epigenetic, depend on expression of specific pattern-determining genes, such as the homeobox gene Xhox3, which is expressed in an anterior/posterior (cranial/caudal) gradient (Ruiz I Altaba and Melton, 1989a,b). Heterotopic transplantations and a culture system for manipulating myotome development *in vitro* would help resolve these questions.

Two other unresolved issues are the fate of primary myotome muscle fibers in the trunk and tail and the origin of multinucleated secondary myotome fibers. The first could perhaps be studied if a marker were available for cells undergoing programmed cell death. The origin of secondary myotome myoblasts and the fate of trunk primary myotome myotubes could be solved by careful transplantation of labeled cells, perhaps using lineage markers such as injected fluorescent dextran (Slack, 1984) or the *Xenopus laevis*/*Xenopus borealis* transplantation system (Thiebaud, 1983).

The diversity of strategies for aligning myotomal fibers (rotation, sorting out, rosette formation) and for achieving multiple copies of DNA and multiple nuclei (polyploidy, fusion, amitosis) raises another interesting question. What evolutionary selection forces produced such astonishing variety in the early, essential events of muscle formation? One attractive hypothesis is that selection for faster rates of development produces heterochronic muscle formation (Blackshaw and Warner, 1976; Forman and Slack, 1980). That is, species that develop rapidly produce muscle at relatively earlier developmental stages than slower developers. If this is true, then it is clear that an entire program for myogenesis has not been moved *in toto* to earlier or later stages. Instead, nearly all the steps of myogenesis become modified or rearranged in time to suit the requirements of each species.

As a possible example, consider *Xenopus laevis*, which develops rapidly compared to other species of amphibia. Muscles first twitch at stage 24, about 24 hours after fertilization, and embryos hatch at stage 34/35, a day later. In contrast, *Ambystoma mexicanum* axial muscles do not function until stage 32, which takes five days, and hatching does not occur until stage 42 at 12 days. If one compares the developmental rates of *Xenopus laevis* with *Ambystoma mexicanum*, corrected for temperature, *Xenopus laevis* develops to an equivalent stage in approximately one fourth of the time. Furthermore, one could hypothesize that many features of *Xenopus laevis* myogenesis are adaptations for this accelerated, precocious myogenesis. A list of the features identified thus far includes:

1. Early commitment of cells to become myoblasts.
2. Synthesis of sarcomeric proteins soon after myoblast commitment and before myotome segmentation.
3. Rotation of myoblasts through 90 degrees and then differentiation of mononucleated cells, thereby avoiding the proliferation, aggregation and fusion steps in making multinucleated myotubes.
4. Polyploidization of muscle nuclei, thus increasing the number of DNA copies available for transcription of sarcomeric specific messages without going through mitosis.
5. Differentiation of cranial myotomes. This advances the stage at which muscles become functional, because innervation and differentiation proceeds in a craniocaudal direction.
6. Electrical coupling between adjacent myotomes. This permits caudal myotomes to function early, even though the cranial spinal nerve reaches only the most cranial differentiated myotome. In *Xenopus laevis* myotomes are electrically coupled, whereas in *Ambystoma mexicanum* they are not (Blackshaw and Warner, 1976).

This hypothesis predicts that there is a direct relationship between the rate of development and modes of PMM myogenesis. Fast-developing amphibians may share common features of myogenesis that differ from those of slow-developing amphibians. Amphibians that develop slowly and produce functional muscle late should show characteristics similar to slow-developing amniotes. Therefore, direct developing amphibians such as *Eleutherodactylus martinicenis* would be expected to show amniote-like myotomogenesis (as suggested by Muntz, 1975).

Differentiation of fiber types

Up to this point we have considered the origin and fate of muscle cells but have said relatively little about the differentiation of muscle fibers. This area in particular demonstrates the diversity of developmental histories because the identity of fiber types and the timing of their appearance differs from species to species, and generalities are difficult to formulate. On firmer ground

TABLE 1

IDENTIFICATION OF AMPHIBIAN SKELETAL MUSCLE FIBERS

Amphibian Fiber Type[1]	ATPase Activity[2]			Other Enzyme Activity[3]				Force/		Amniote Fiber Type
(Anuran)	Alkaline	Acid I	Acid II	NADH	GPDH	PP	SDH	Velocity[4]	Diameter[5]	Homolog[6]
larval white	+	-	-	-	-	+++	+	-	L	?
larval red	+++	-	-	-	-	+	+++	-	S	?
1	+++	0	+	+	+	+++	++	F/TW	L	IIB
2	+++	++	+	++	++	++	+++	F/TW	L	IIA
3	+++	++	+	+++	+++	++	++++	S/TW	S	I
4	+	+	+	+	+	+	+++	VS/TW	S	?
5	0	-	-	-	-	-	0	S/TO	S	?
(Urodele)[7]										
Larval white	+++	+	+	-	-	-	+	-	L	
Larval red	+++	+	+	-	-	-	+++	-	S	?
1	+++	++	0	-	-	-	-	-	L/M	IIB
2	+++	0	ND	-	-	-	-	-	L/M	IIA
?	+++	++	+	-	-	-	-	-	S	IIC
3	+	+++	+++	-	-	-	-	-	S	I

[1]Amphibian fiber types are identified by appearance, function, and histochemical assays. In the left column are anuran fiber types identified and numbered by Smith and Ovalle (1973) and Watanabe *et al.* (1978). Characteristics in columns to the right are combined from several studies of various species. Because not all the histochemical assays have been done for each fiber type in each species, discordances may exist. [2]Myosin ATPase: histochemical activity after preincubation at alkaline (greater than 9.4), acid I (4.5-4.6), or acid II (4.2-4.3) pH. Number of pluses indicates the relative intensity of the reaction product, minus means the assay has not been done. [3]Relative intensities of reaction product after histochemical assay for: NADH (nicotinamide adenine dinucleotide diaphorase), GPDH (glycerophosphate dehydrogenase), PP (phosphorylase), SDH (succinic dehydrogenase) (Bretones *et al.*, 1987; Sperry, 1981). [4]Force/velocity of contraction measured directly in individual isolated fibers (Lannergren and Hoh, 1984). F/TW, fast twitch; S/TW, slow twitch; VS/TW, very slow twitch; S/TO, slow tonic. [5]Relative fiber diameter: (L)arge, (M)edium, (S)mall. [6]Amniote fiber type homologue as predicted from the classification scheme of Brooke and Kaiser (1970) [7]Urodeles fibers are classified in this table according the nomenclature for anurans, using the characteristics reported by Chanoine *et al.* (1987), and Watanabe *et al.* (1980).

is our understanding of the molecular basis for gene expression in muscle cells. New technologies for analyzing gene expression in amphibians offer potentially even more rapid advances in understanding muscle cell differentiation.

How many muscle fiber types have been identified in amphibia, and how do they compare with amniote fiber types?

The number of fiber types identified in amphibia depends on the criteria used to characterize fibers. Early work, based primarily on histological appearance, revealed three skeletal fiber types: red, white, and intermediate. More recently, two classification systems have been used to identify mammalian and avian fiber types, based on either myosin ATPase pH lability, (Brooke and Kaiser, 1970) or metabolic activity (eg. Peter *et al.*, 1972). When these criteria are applied to amphibians, up to five fiber types can be distinguished in adult skeletal muscle (Table 1). However, the two systems do not always agree because the relative levels of histochemical activities used to identify fiber types are not consistent between taxonomic classes. Thus, for example, mammalian fast IIB fibers have high NADH activity, whereas amphibian IIB fibers, as identified by myosin ATPase pH lability, have low NADH activity (Burgos-Bretones *et al.*, 1987). This amount of "biochemical noise" is not surprising given the evolutionary distance separating amphibians and amniotes and their different requirements for muscle function (and differences between laboratories in interpreting histochemical assays). Through evolution, homologous myosin isoforms with similar pH optima may come to occupy myofibers with different physiological roles and hence different metabolic profiles. This difficulty in matching mammalian and amphibian fibers one-to-one has led some to propose an entirely different nomenclature for amphibian fiber types (Lannergren and Smith, 1966; Engel and Irwin, 1967; Smith and Ovalle, 1973). However, rather than create a separate nomenclature for amphibians, it seems reasonable for comparative purposes to adopt the widely used fiber type terminology of higher vertebrates, keeping in mind that not all the characteristics of one fiber type will apply to all species, and noting differences when they become relevant (eg. Chanoine *et al.*, 1987). This has been done in Table 1.

Are there stage-specific fiber types?

Because of the difficulties in defining and identifying fiber types discussed above, this question still awaits an answer. The data at present, however, suggest that at a minimum, embryonic or tadpole stage fibers differ from those found in adults.

The first fibers to differentiate are those of the myotome. Histochemical staining and morphology identify

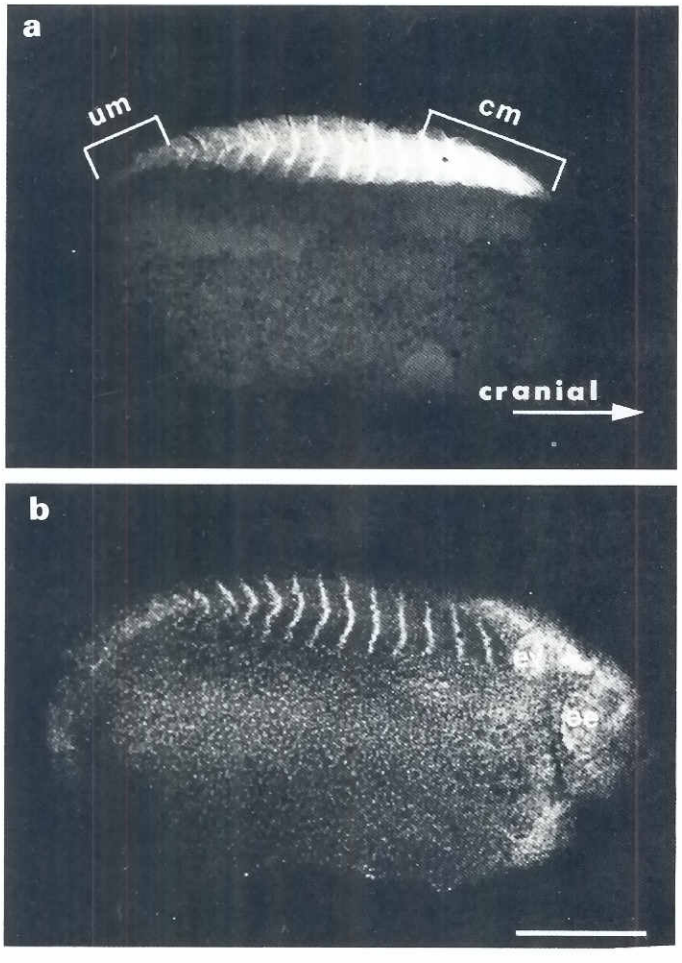

Fig. 4. Cranial/caudal differentiation of *Xenopus laevis* myotomes visualized with anti-actin antibody. *At this stage (23/24) myotomal muscles first become functional.* **(a)** *Cranial myotomes (cm) stain more intensely for actin than caudal myotomes, and the cranial and caudal borders of each myotome stain more intensely than the centers. Note also that actin is expressed in unsegmented paraxial mesoderm (um). Whole-mounted embryo was fixed in methanol and stained with anti-sarcomeric actin monoclonal antibody HUC 1-1 (kindly provided by J. Lessard) followed by FITC labeled secondary antibody.* **(b)** *The same embryo treated with Hoechst stain reveals the location of cranial myotomes with respect to the otic vesicle primordium (ov) and the developing eye (e). Myocyte nuclei are in register at the centers of each myotome. Magnification bar = 500 lm.*

these first myotubes as similar in some respects to fast skeletal fibers, probably equivalent to mammalian type IIB, but of an "embryonic" type. In *Xenopus* they become functional at stage 24, shortly after neurulation, and are the only fiber type detected in myotomal muscle until about stage 46, before metamorphosis, when slow twitch fibers can first be detected. A similar pattern appears in urodeles, which have a single "larval" fiber type until just before metamorphosis, when slow fibers can be identified (Table 1). As in other lower vertebrates, slow twitch fibers in *Xenopus* and other anuran tadpoles are long, thin, and arranged in a layer around a core of fast fibers in each myotome. (Flood *et al.*, 1977; see Kordylewski, 1986 for review). There are relatively more slow fibers in the caudal myotomes than in the cranial ones (Kordylewski, 1986; Schwartz and Kay, 1988). An interesting exception to the general rule that slow fibers are peripheral to fast fibers in larvae is found in some urodeles, where fast and slow axial fibers are mixed, as they are in adult muscle of all amphibians (Watanabe *et al.*, 1980; Chanoine *et al.*, 1987). In the leg, fibers are mixed from their first appearance, whereas in axial muscle the pattern changes from peripheral to mixed by an undetermined mechanism, perhaps by proliferation and differentiation of dispersed myoblast (satellite) cells of different lineages (See Fig. 1).

Adult fiber types probably first begin to appear during metamorphosis. This is most obvious in the leg, which of course does not exist before metamorphosis, but it is also true in axial muscles. In *Xenopus*, both fast and slow fibers can be detected in metamorphosing leg muscle (Kielbowna, 1980). These fibers express N-CAM, in contrast to myotomal muscles (Kay *et al.*, 1988). After metamorphosis, at least five fiber types can be distinguished in adult leg muscle (Table 1), but when these additional three fibers first appear during development is not known. In urodeles, adult fibers also appear during metamorphosis, as well as an intermediate fiber type (IIC) specific to metamorphosis. Perhaps this type is equivalent to the neonatal type found in amniote skeletal muscle (Chanoine *et al.*, 1987).

What regulates fiber changes induced at metamorphosis?

It has been known for 80 years that thyroid hormones induce amphibian metamorphosis (Fox, 1983). Recently, it has become clear that thyroid hormones also have profound effects on muscle gene expression (Izumo *et al.*, 1986). However, there have been surprisingly few studies of the role of thyroid hormones in amphibian muscle development. In a recent experimental analysis in urodeles, a rise in circulating thyroid hormone level was found to be correlated with a change in fiber type, as judged by myosin ATPase pH lability, and also with a change from larval to adult myosin isoforms (Chanoine *et al.*, 1987). Hypophysectomy or treatment of larvae with thiourea to induce hypothyroidism and prevent morphological metamorphosis also prevented the appearance of adult fiber types and adult myosin isoforms. When thiourea treatment was stopped, adult fiber types and adult myosins appeared. However, when metamorphosis was induced precociously with triiodothyronine, adult myosin isoforms appeared early, but surprisingly, adult fiber types, as assayed by myosin ATPase pH lability, did not. This result demonstrates the limitations of histochemical identification of fiber types and points to the need for more sensitive and reliable techniques, such as RNase protection assays

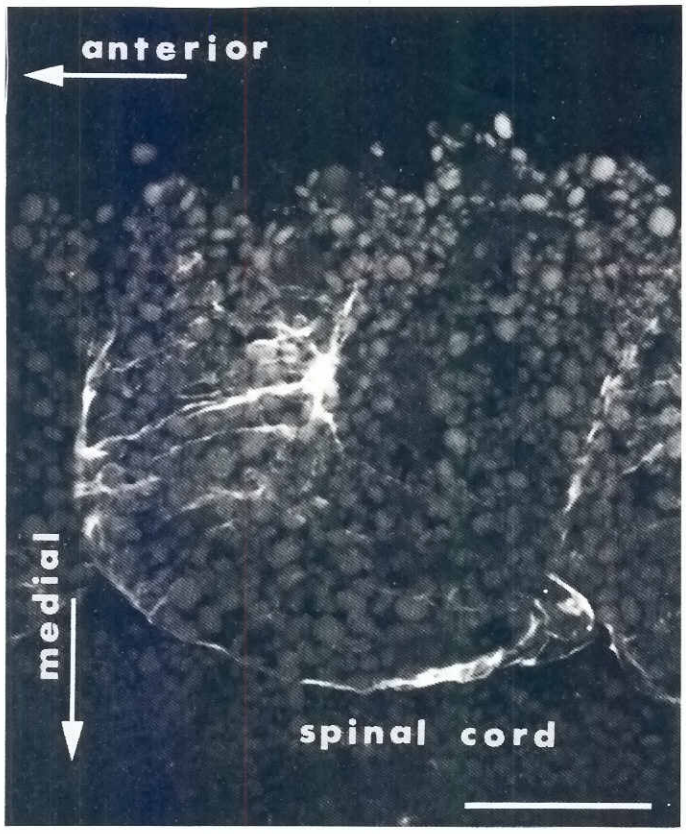

Fig. 5. Transient polar localization of sarcomeric actin in differentiating *Ambystoma mexicanum* myotome. *Actin first appears at the periphery of individual mononucleated myocytes in the anterior (cranial) and medial regions of the myotome. Subsequently, these cells fuse. The resulting multinucleated myotubes span the length of each myotome and actin appears throughout the myotome fibers. Oval structures are autofluorescent yolk platelets. Optical section of a proximal tail myotome from a stage 35/36 embryo. Embryo was formaldehyde fixed, permeablilized with methanol, and stained with antibodies as described in Fig. 3. Image was obtained with a scanning laser confocal microscope. Magnification bar = 50 lm.*

for known messenger RNAs (Mohun *et al.*,1984) to monitor changes in muscle differentiation.

The proportion of fiber types within certain adult muscles can also be regulated by androgens. Sexual differentiation after metamorphosis results in a male larynx that is almost all fast twitch (type IIA) fibers, whereas in females it is a mixture of slow twitch and intermediate fibers (Type I and IIC). This difference apparently arises from selected proliferation of satellite cells rather than from switching of myosin isoform expression in previously existing fibers. It is not reversible after about 6 months from metamorphosis. (Sassoon *et al.*, 1987).

When does muscle gene expression begin?

Two extreme patterns for the timing of muscle gene activation have been discovered so far. The first pattern appears in *Xenopus*, where muscle-specific α-actins can be detected extraordinarily early in development, in late gastrula at about 10 hours of development (Ballantine *et al.*, 1979; Sturgess *et al.*, 1980; Mohun *et al.*, 1984; Gurdon and Cascio,1987). Differentiation proceeds cranially to caudally, as is readily seen by staining whole embryos with fluorescent antibodies against actin (Fig. 4). In addition to α-actin synthesis, it is now known that at least two muscle-specific myosin heavy chain (myHC) transcripts are also synthesized at late gastrula stage (Radice and Malacinski, 1989). Like the muscle actins, these myHC's increase in abundance until metamorphosis, but, unlike the actins, they decline during metamorphosis and are reduced or eliminated in adults. They may be homologous to the embryonic fast muscle isoform found in amniotes (Whalen *et al.*,1979; Strehler *et al.*,1986). This precocious synthesis anticipates the early function of primary myotomal myocytes at the end of neurulation, which occurs in *Xenopus* at about 24 hours of development.

The other extreme pattern of expression in time has been found in the axolotl. In this species, actin and myosin first appear later in development, at late tailbud, just prior to myocyte fusion and innervation (Forman and Slack, 1980; Neff *et al.*, 1989).

In the two urodeles studied, *Ambystoma mexicanum* and *Cynops pyrroghaster*, differentiation proceeds with transient patterns of anterior/posterior and medial/lateral expression of sarcomeric actin and myosin (Fig. 5; see also Neff *et al.*, 1989). Moreover, in contrast to *Xenopus*, *Ambystoma* sarcomeric actin and myosin are not expressed until after somite segmentation (Forman and Slack, 1980; Neff *et al.* 1989).

The actins expressed early in development continue to be expressed during metamorphosis and in adults. In contrast, myHC isoforms expressed early are replaced later by adult isoforms. This has been demonstrated in *Xenopus*, where an adult skeletal myHC transcript first appears during metamorphosis. Curiously, it is found in both developing leg and degenerating tail muscle (Radice and Malacinski, 1989). Apparently the signal, perhaps thyroid hormone, that triggers expression of the adult isoform in leg fibers also operates in tail muscle, even though an apparently separate mechanism causes similar cells to die. Perhaps this same mechanism also initiates the expression of N-CAM in leg muscle, but not myotomal muscle (Kay *et al.*, 1988).

How many isoforms of sarcomeric actin and myosin are expressed in embryonic muscle?

There appear to be about 6-8 actins in *Xenopus*, at least three of which are expressed early in muscle development. Urodeles apparently express approximately the same number (Vanderkerckhove and Weber, 1984).

As in other vertebrates, both the cardiac and skeletal isoforms of α-actin are found in primary myoblasts (Mohun *et al.*, 1984). Their isoform identity is based on the aminoterminal amino acids found at positions 2 and 3, which characterize cardiac and skeletal isoforms in all other vertebrates examined (Vanderkerckhove and Weber, 1984). In contrast to what happens in other vertebrates, however, co-expression of α-actin isoforms continues through late tadpole stages and persists in both adult skeletal muscle and in heart (Vanderkerckhove and Weber, 1984). Thus, in *Xenopus*, complete tissue-specific regulation does not occur. Whether the two actin isoforms are expressed by different myofibers within the muscle mass or by the same myofibers is not known.

In addition to the two α-actins just described, *Xenopus* also contains a processed α-actin gene that does not contain introns (Stutz and Spohr, 1987). Interestingly, in *X. laevis* this processed gene is expressed in tadpoles and adult skeletal muscle, whereas the homologue in the related species *X. tropicalis* is expressed in tadpoles and adult heart. Furthermore, in *X. tropicalis* the gene contains a substitution that translates into an amino acid replacement of Asp with Glu at position 3. Glu at this position is characteristic of all other vertebrate cardiac actin genes (Vanderkerckhove and Weber, 1984). Thus, not only is this gene expressed in different tissues in the two species, but through evolution it has acquired mutations that conform to other actin sequences specific for heart or skeletal muscle.

Finally, *Xenopus* expresses three cytoplasmic actin genes (Vanderkerckhove and Weber, 1984; Cross, *et al.*, 1988). One of these is unusual because its expression is restricted to muscle cells (Mohun and Garrett, 1987).

Among urodeles, in *Pleurodeles watlii*, a single isoform of actin appears in both heart and skeletal muscle (Vanderkerckhove and Weber, 1984; Khrestchatisky and Fontes, 1987). If a single isoform appears in other urodeles as well, this will lend support to the polyphyletic origin of the urodeles and anurans (Jarvik, 1980).

The number of amphibian myHCs is unknown, but genomic Southern blot analysis suggests there are approximately 8-10 genes in *Xenopus* (G.P. Radice, unpublished). A slightly larger number may exist in urodeles as estimated by protein electrophoresis (Chanoine *et al.*, 1987). Myosin heavy chain expression has been studied in the axial skeletal muscle of *Pleurodeles watlii, Ambystoma mexicanum*, and *Ambystoma tigrinum* (Chanoine *et al.*, 1987). These animals have three larval and three adult specific axial myHCs. In addition, all have myHC's that appear during metamorphosis but are not expressed in adults. These "transitional" isoforms may be similar to the neonatal myHCs found in amniotes, which are first expressed at birth or hatching (Stockdale and Miller, 1987).

What regulates the spatial and temporal expression of muscle genes?

A combination of cis-acting DNA sequences and trans-acting protein factors is commonly invoked to explain the regulation of muscle gene expression. One particularly well-studied example of a cis-acting regulatory sequence is the CArG box (CC(A/T)GGG). This sequence has been shown to be sufficient and necessary for actin gene activation in vertebrates such as human (Miwa and Kedes, 1987; Gustafson *et al.*,1989), rat (Nudel *et al.*, 1989), mouse (Hu *et al.*, 1986), and chicken (Grichnik *et al.*, 1988; Schwartz *et al.*, 1989). In *Xenopus*, four copies of the CArG box are present in the cardiac actin gene in a region from -412 to -70 upstream of the transcription start site. The most proximal CArG box (CArG box 1, -80) is essential for muscle-specific gene expression (Mohun *et al.*, 1986, 1989) and also for correct temporal expression (Gurdon *et al.*, 1989).

Much attention has focused on the CArG box because it shares extensive homology with the serum response element (SRE) present in the human c-fos and in *Xenopus* cytoskeletal actin gene promoters (Mohun *et al.*, 1987). CArG box and SRE are functionally interchangeable in transient expression assays (Taylor *et al.*, 1989). Moreover, both sequences can form a complex with a serum response factor (SRF) or a very closely related protein (Taylor *et al.*, 1989). No doubt these SRF-like substances play an important role in regulating actin gene expression. However, SRF is expressed later in mouse development than the first appearance of actin, and the CArG box factor is found in non-muscle cells (Boxer *et al.*, 1989). This mechanism alone cannot account for the correct developmental stage-specific expression of actin genes. There must be additional unknown factors or mechanisms involved in activating actin expression. These may include regulation by activation of DNA binding proteins or cooperative interactions of several factors in cell-type-specific ways, as suggested by Taylor *et al.* (1989).

Nothing is known about sequences responsible for myosin gene regulation in amphibians. One might expect that they would be coordinately regulated by some of the same factors that activate actin genes. Thus, it has been quite surprising that in other vertebrates there seem to be no common regulatory sequences in the promoter regions of the actin and myosin heavy chain genes, although there are CArG-like sequences in the myosin light chain genes of mouse (Barton *et al.*, 1989). Instead, myosin heavy chains appear regulated by concerted action of multiple positive and negative regulatory elements (Bouvagnet *et al.*, 1987), including cis-acting promoter sequences that bind thyroid receptors (Mahdavi *et al.*, 1989). It appears that coordinate expression of muscle-specific genes will be regulated by the interactions of multiple transcription factors, few of which will be individually speci-

fic to muscle cells but will be selectively activated and combined to produce distinct patterns of gene expression and hence generate distinct muscle phenotypes.

Which protein isoform transitions are autonomous (lineage regulated), which are hormonally regulated and which are regulated by innervation?

Amphibian primary myocytes differentiate autonomously in culture and in the embryo before innervation. Expression of cardiac and skeletal α-actin and of embryonic myosin heavy chain is therefore autonomous in these cells. Myosin heavy chain isoform switching that occurs at metamorphosis probably requires thyroid hormone, since in amniotes expression of all of myHC genes is regulated by thyroid hormone (Izumo *et al.*, 1986; Swynghedauw, 1986). Parvalbumin appears in *Xenopus* fast twitch muscle after the expression of actin and myosin has begun and after myofibers have become innervated. However, its expression does not require functional nerve because it is expressed in animals raised in the presence of lidocaine to block signal transduction (Schwartz and Kay, 1988).

Prospects and perspectives

There is much to learn about muscle gene expression in amphibians. At one level, much work needs to be done identifying the number of genes and isoforms of muscle proteins expressed during development. The list apparently is complete for *Xenopus* actins, but for myosin heavy and light chains, and other sarcomeric proteins, there are large gaps. In particular, it will be important to learn the number and pattern of expression of myHCs. Is there an isoform homologous to amniote neonatal fast skeletal isoform? Studies in urodeles suggest there is (Chanoine *et al.*, 1987). Are there cardiac-specific myHCs? Again in urodeles, an isoform expressed in both cardiac and leg muscle has been found, similar to the cardiac β/slow muscle isoform of amniotes (Casimir *et al.*,1988). The list, however, is obviously incomplete and needs to be filled in before a coherent picture of amphibian myogenesis can be attained.

What is the adaptive value of expressing so many protein isoforms at various developmental stages? For the myosins, different myosin heavy chains confer on myofibers different ATPase activities (Staron and Pette, 1987). Diverse ATPase activities may be part of how evolution fine-tunes muscle fibers for a particular activity (see also Peters, 1989). Alpha-actins, on the other hand, appear functionally identical *in vitro*, can coassemble in cells after transfection and are normally coexpressed in early myofiber differentiation (Wade and Kedes, 1989). But there may be subtle differences among actins that await discovery. A hint in this direction is the observation that the few amino acid differences between cardiac and skeletal α-actin occur in a region of myosin binding. Thus, actin isoforms may be specialized for efficient interaction with myosins (Sutoh, 1982).

Part of the mechanism for controlling the pattern of muscle gene expression appears to involve thyroid hormone. The mechanism of thyroid hormone action is still obscure, so there are many unanswered questions. For example, how does thyroid hormone simultaneously cause the degeneration of tail muscle and trigger development of leg muscle? Do thyroid hormones act directly on muscle cells or indirectly, perhaps by first acting on nerve or connective tissue cells? Does thyroid hormone cause fiber type switching by changing isoform expression in existing fibers, or by inducing selective death of some fibers and proliferation of new myoblasts, or both? What is the mechanism that permits a brief hormone treatment at the larval stage to have its effect on fiber type development months later? Much of the specificity of response to thyroid hormones apparently lies in selective interactions of thyroid hormone receptors with specific regions of DNA (Umesono and Evans, 1989). Because of the ease with which metamorphosis can be manipulated, hormonal regulation of amphibian myogenesis seems a particularly rich and promising area for future investigations.

An ideal approach for studying developmental gene expression is to generate developmentally interesting mutations. Unfortunately, this is impractical in amphibians because of long generation times. An alternate approach is to clone the putative regulatory regions of muscle-specific genes and reintroduce them into embryos, creating transgenic animals. Although a premier feature of amphibians is the ease with which altered genes can be injected into the large eggs, there have been two frustrating difficulties. The first is that most of the injected DNA is not integrated into chromosomes, but replicates extra-chromosomally until the gastrula stage, after which it mysteriously stops replicating and gradually is eliminated from the embryo (Rusconi and Schaffner, 1981; Bendig and Williams, 1983; Forbes *et al.*, 1983). This problem is somewhat alleviated for genes expressed early because, with appropriate promoters, genes will be transcribed and translated before they disappear (Etkin and Balcells, 1985; Kreig and Melton, 1985; Krone and Heikkila, 1989). A more difficult problem for those interested in the spatial regulation of genes is that expression of genes injected into eggs is mosaic. Apparently, the DNA is not distributed evenly to daughter blastomeres, so that not every cell receives the foreign DNA (Forbes *et al.*, 1983; Etkin and Pearman, 1987). Furthermore, there is considerable cell mixing during blastula and gastrula stages, so that the few transformed cells probably become dispersed. (Heaysman *et al.*, 1984; Wilson *et al.*, 1986; Sheard and Jacobson, 1987). Because of these persistant technical problems, the possibility of creating transgenic amphibians using sperm to introduce foreign DNA at fertilization has caused much excitement (Lavitrano *et al.*, 1989).

If this technique proves reliable it should greatly speed the analysis of presumed regulatory DNA sequences.

In any case, there are sufficient embryological, cellular and molecular techniques currently in hand to move forward rapidly with the task of understanding myogenesis. As stated at the outset, amphibians have much to offer, particularly for studying early stages of induction and commitment to myogenic pathways, but also for analyzing of hormonal control of muscle remodeling. Much of this work is likely to focus on *Xenopus laevis* as a test species. Although conventional genetic approaches to developmental analysis are not feasible, the apparent diversity of amphibian muscle developmental histories provides a wealth of "experiments of nature", and comparisons of myogenic mechanisms among different amphibians should be exploited. Rapid advances in molecular biological techniques should make such comparative studies easier and provide exciting new information soon.

KEY WORDS: *amphibian, myogenesis, mesoderm, muscle*

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