

Gene Regulation in Muscle Development and Control of Myogenic Lineage by Determination Genes

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Introduction

The mechanism underlying the elaboration of diverse cell types in vertebrates is a major area of inquiry of developmental biology. Skeletal muscle is an excellent system for such analysis. The elaboration of the contractile apparatus and the specialization of the membrane at the neuromuscular synapse are two features of muscle development that set it apart from other tissues. Over 40% of a mammal's mass is skeletal muscle (Burleigh, 1974). Hence, muscle differentiation yields the largest tissue mass of the organism. In man, this means that $\sim 10^{12}$ nuclei are committed to the expression muscle-specific genes. And each of the trillion or so nuclei arose from a single stem cell in the beginning – the fertilized egg that gave rise to all the other specialized cell types of the adult organism as well.

In what follows, broad features of myogenesis are outlined at the molecular level. This review is by no means complete; rather, it is meant to serve as a primer of the molecular biology of muscle development in particular and gene regulation in general. Where possible, reviews are cited and more complete references to the primary literature can be found there.

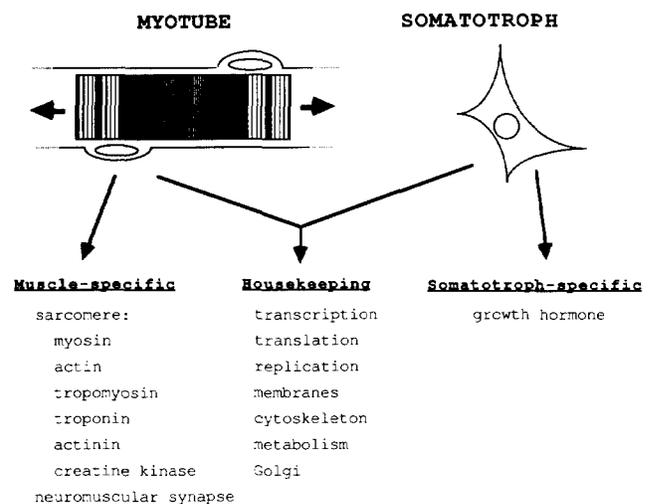
Differential Regulation of Genes During Development

Stated in simple terms, the developmental problem can be defined at the level of the gene as follows: The diploid genome of a fertilized egg gives rise to over several hundred different cell types comprising the various tissues of the adult organism. Each cell type contains the same genetic information (i.e., DNA sequences) but uniquely expresses one or more genes encoding tissue-specific proteins; that is, polypeptides found only in that tissue. Figure 1 illustrates this in general terms for a multinucleated myotube and a somatotroph of anterior pituitary. Myotubes express a large set of genes for muscle-specific proteins of the sarcomere (skeletal actin, myosin, tropomyosin, troponin, actinin, etc.), and the neuromuscular junction (membrane proteins, acetyl-

choline receptor, etc.); it also expresses genes for polypeptides involved in the specialized metabolism of muscle in the sarcoplasm (creatine kinase, myoglobin). By contrast, somatotrophs express only one tissue-specific gene and it encodes growth hormone.

Tissue-specific proteins have also been termed "luxury" products to convey the idea that the proteins serve the needs of the organism rather than the needs of individual cells (Holtzer et al., 1969). That is, individual cells can remain viable without expressing the genes for growth hormone and skeletal muscle because both cells, and all other cells of the body, express another set of genes – the "housekeeping" genes that are required for the viability of the cell (Holtzer et al., 1969). Thus, myotubes and somatotrophs express a common set of genes encoding proteins required for cellular metabolism, protein secretion and metabolite transport; for the structure of the plasma membrane, the endoplasmic reticulum, the nuclear membrane and pores and the Golgi apparatus; and for the machinery for the synthesis of proteins (translation), RNA (transcription) and DNA (replication and repair). The number of housekeeping genes is very large, well into the thousands. Over 200 genes, for example, are required for the translation machinery of the cell alone (e.g., ribosomes, tRNAs, translation factors, aminoacyl synthetases, etc.).

Figure 1



Expression of housekeeping and tissue-specific genes in a skeletal myotube and a somatotroph of anterior pituitary.

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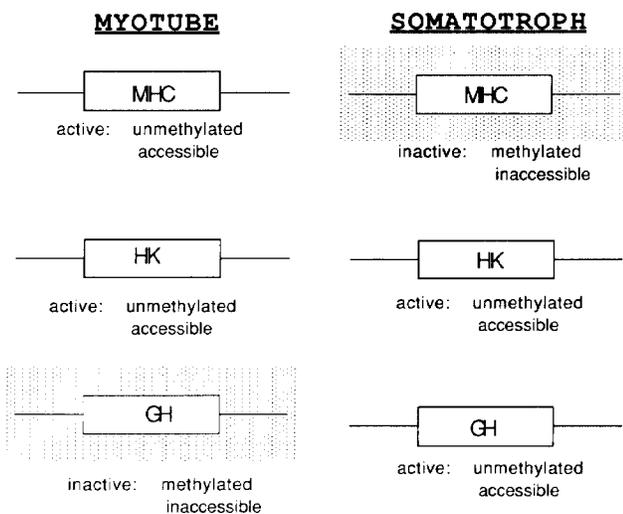
Reciprocal Meat Conference Proceedings, Volume 41, 1988.

In adult tissue, therefore, individual cells express two classes of genes encoding two different kinds of information-cellular (housekeeping) and organismal (luxury or tissue-specific). And it is the differential expression of "tissue-specific" genes that is the hallmark of developmental regulation at the level of the gene. Figure 2 emphasizes this feature more explicitly for a myosin heavy chain gene (MHC) and the growth hormone gene (GH). The MHC gene is expressed at high levels in myotubes but is kept in a silent state in somatotrophs. Conversely, the GH gene is active in somatotrophs and inactive in myotubes and all other cells of the organism. Yet both cells share the property of expressing the very large set of housekeeping genes (HK).

It is worth noting that the level of expression between the active and inactive states of genes can be enormous. For example, we showed that the growth hormone gene is expressed at a level that is 100 millionfold higher in pituitary cells than in liver cells (Ivarie et al., 1983). In fact, this was a minimal estimate limited by the sensitivity of the assay used in the measurement. Thus, the gene could well be completely silent in liver cells. This level of regulation contrasts with the thousand-fold or so regulation achievable in a bacterial operon such as the *lac* operon where a single protein (the repressor) acting at a single DNA site (the operator) blocks transcription of the genes in the operon (Beckwith, Zipser, 1979). Hence, developmental regulatory events can exert powerful effects on the ability of a gene to be transcribed or not.

How is it then that cells with the same genetic information acquire the ability to express one gene but not others during the generation of cellular diversity? As will be discussed in more detail below, considerable attention has focused on the fact that genes are usually "marked" by a modification of DNA that conveys a molecular message for the gene to be silent (Rosin, Riggs, 1980; Doerfler, 1983; Cedar, 1988). "Unmarked" or unmodified DNA is actively expressed and has also undergone a substantial change in the structural

Figure 2



Properties and states of expression of genes for a myosin heavy chain isoform (MHC), a housekeeping protein (HK), and growth hormone (GH) in myotubes and somatotrophs.

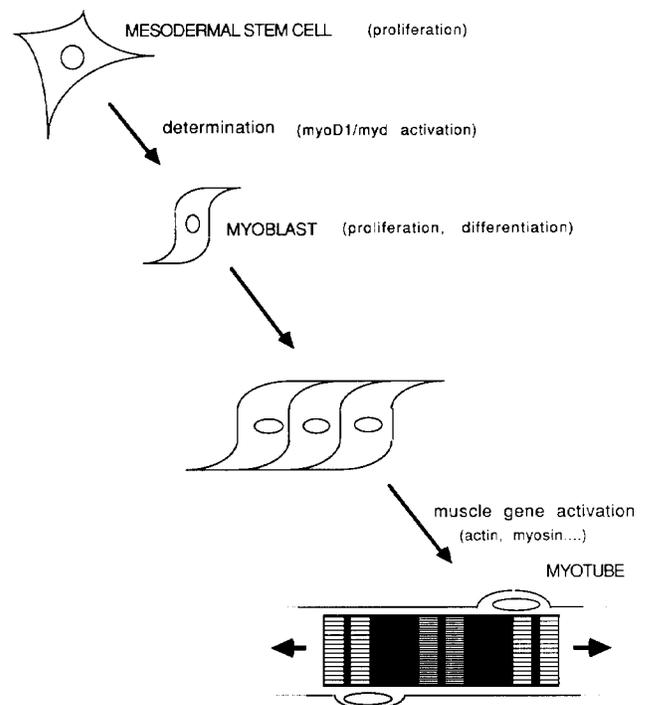
organization in chromatin, making it more accessible to the transcriptional machinery of the cell (Weintraub, 1985). The modification involves methylation of cytosine residues catalyzed by an enzyme (DNA methyltransferase) that uses S-adenosyl-L-methionine as a methyl donor. DNA methylation and the chromatin organization of genes appear to be important features of the regulation of genes during development.

Myogenesis at the Cellular Level

At a cellular level, myogenesis can be broken into two general steps (Figure 3). First, a mesodermal cell is converted to a proliferating myoblast. Second, myoblasts fuse to form a multinucleated myotube in which muscle-specific genes have been activated. In the first step, *determination*, cells and all their descendants are committed to the myogenic lineage. In the second, *terminal differentiation*, myoblasts form nondividing myotubes and activate the set of muscle-specific genes.

Note that steps leading to the origin of the mesodermal cell have not been included. Although little is known about the molecular biology of these steps, developmental biologists have worked out many of the details of the cellular pathway to skeletal muscle in the early embryo (Patten, Carlson, 1974; Saunders, 1982; Reddington, 1983). Skeletal muscle derives from the middle or mesodermal layer of the three germ cell layers (ecto-, endo- and mesoderm), that give rise to the organism. Mesoderm is a pluripotent tissue that differentiates into a multitude of cell types besides skeletal muscle, including bone, cartilage and cardiac muscle; parts of the eye, teeth, kidney and gonads; and the many cells of the hematopoietic pathway (erythrocytes, lympho-

Figure 3



Outline of steps associated with myogenesis at a cellular level.

cytes, macrophages, etc.). Mesoderm is also identified by where it occurs in various regions of the early embryo (dorsal, head, lateral) and it is dorsal mesoderm that produces morphologically visible somites and myotomes from which skeletal muscle differentiates. Determination, therefore, appears to involve conversion of dorsal mesodermal cells to proliferating myoblasts, thereby committing the mesodermal cell and its descendants to a myogenic lineage. As discussed at the end, two genes acting at or near this step have now been identified using an established tumor cell line of mesodermal origin.

An interesting feature of myogenesis is that myoblasts can self-renew as well as differentiate terminally into nondividing myotubes. Muscle differentiation is a protracted process occurring from early embryogenesis to adulthood (Burleigh, 1974). In the rat, for instance, myotubes are detectable as early as day 12 of development and continue to be produced for >15 weeks after birth. The capacity for self-renewal ensures that progenitor cells are always available throughout this long period to build necessary muscle mass.

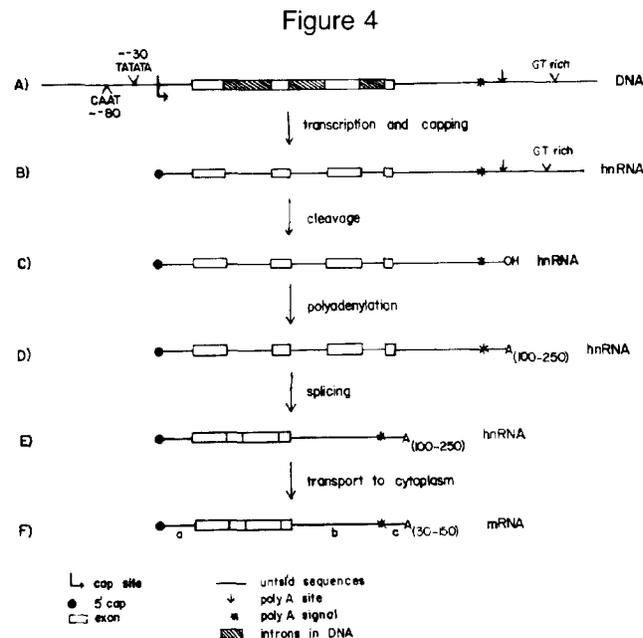
It is also important to note that Figure 3 represents an oversimplification and underemphasizes other steps associated with later stages of terminal differentiation. For instance, fusion is shown as preceding activation of muscle gene transcription. However, skeletal muscle gene activation can occur without cell fusion (Endo, Nodal-Ginard, 1987) just as it does in cardiac muscle development. Thus, fusion is not a requisite signal for gene activation. Also, as discussed in more detail below, there are additional switches in muscle gene expression that occur as the organism matures from fetus to adult. For instance, the MHC genes form a gene "family" that are expressed sequentially at various stages of myogenesis and in different muscle types as isoforms are incorporated into myofibrils (Mahdavi et al., 1986). Thus, the progression in MHC gene expression from embryonic to adult isoforms could have been added to Figure 3 as a maturation step after myotube formation. Stage- and muscle-specific regulation is discussed in more detail after the next section.

From Gene to Phene: An Average Mammalian Gene and Its Expression

Before proceeding to the establishment of the muscle cell phenotype by activation of muscle-specific genes, it may be useful to review the structure of a "typical" mammalian gene and the molecular events underlying its expression into a mature messenger RNA that can be translated in the cytoplasm. These are diagrammed in Figure 4.

Perhaps the most remarkable finding in the molecular biology of eukaryotes occurred in the 1970's when it was found that coding sequences of most vertebrate genes are interrupted with noncoding DNA. On average, a gene's coding sequence is interrupted 5 to 10 times with noncoding DNA. The interrupting sequences are called "introns" for "intervening" sequences and the coding segments arising from the interruptions are called "exons" for "expressed" sequences. Note that not all genes have introns and the number of introns per gene can vary substantially.

Transcription is initiated at a single site (usually) at the 5' end of the gene using short sequence elements immediately



A "typical" mammalian gene and events leading to its expression as a cytoplasmic messenger RNA.

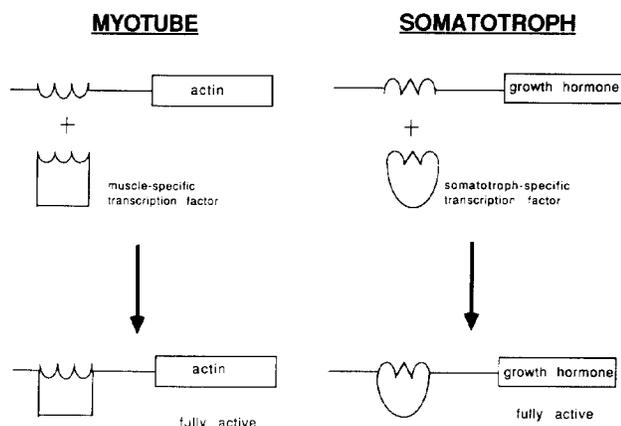
upstream of the first nucleotide of the message. Such elements comprise a gene's "promoter" and for tissue-specific genes, these are usually a TATAA element at ~25-30 bases from the transcription start site and a CCAAT element at ~80-100 bases from the start site (Konieczny, Emerson, 1984). Housekeeping genes usually lack these sequences and use GC-rich elements instead for their promoters. In either case, transcription of both classes of genes is accomplished by RNA polymerase II proceeding from the 5' end through the entire length of the gene and a bit beyond and terminating at the 3' end by the addition of a string of adenine residues, which form the poly-A tail. Shortly after transcription is initiated, the 5' end of the nascent transcript is also "capped" with a methylated guanine linked by 5'-5' phosphodiester bond. Note that while all messenger RNAs contain the cap, which is required for translation of the messenger RNAs, not all messengers contain poly-A tails.

Before transport to the cytoplasm can take place, introns must be removed by a precise process termed "splicing." That is, the 3' end of one exon is joined to the 5' end of the adjacent exon whose 3' end is joined to the 5' end of the next exon. Eventually a messenger RNA results that lacks introns and contains the exons joined in the correct linear order. After transport to the cytoplasm, messages are translated by the translation machinery of the cell into the functional polypeptide which, in turn, may or may not be modified in ways that affect its activity.

Tissue-Specific Transcription Factors

In the past several years, it has become apparent that cells of adult tissues also contain cell type- or tissue-specific transcription factors that assist RNA polymerase II in transcribing accessible genes (McKnight, Tjian, 1986). This is depicted in Figure 5 for a myotube and a somatotroph. The myotube contains a factor interacting with a specific se-

Figure 5



Tissue-specific transcription factors active in skeletal muscle and anterior pituitary somatotrophs.

quence on the skeletal muscle actin gene enhancing its transcription (Muscat, Kedes, 1987). By contrast, somatotrophs lack the muscle transcription factor and contain instead a different factor interacting at a specific region on the growth hormone gene (Nelson et al., 1988). These factors were discovered originally by the observation that a skeletal actin gene, engineered so that it lacked the DNA binding sequence for the factor, was inactive in muscle cells whereas the gene with the DNA sequence was active in muscle but not in nonmuscle cells. Furthermore, placing the sequence in front of a nonmuscle "reporter" gene allows the reporter gene to be expressed in a muscle-specific manner.

A large number of cell type-specific transcription factors have been discovered in this way. The DNA sequences to which they bind are called "enhancers" because of their effect on transcription. Such sequences can lie near promoters or at large distances from them. Often, their orientation can be reversed and they still function normally. As discussed next, such factors and their DNA binding regions are likely to be involved in both muscle type- and stage-specific regulation of sarcomeric protein.

Activation of Muscle-Specific Genes and Isoform Diversity

It is important to keep in mind that the term "gene expression" includes all the steps involved in converting the information in a gene into a functional protein. So regulation can occur at levels other than gene transcription. Perhaps this is best exemplified by the many pathways in which muscles genes are regulated to generate the isoform diversity characteristic of the adult tissue (Emerson et al., 1986). Alterations in promoter utilization as well as changes in splicing pathways play important roles in muscle-specific gene expression. In this section, discussion is limited to effects occurring at the level of the promoter.

Two kinds of regulation occur in muscle at the level of gene activation: stage-specific and muscle type-specific. In the first, genes of a single type but encoding different isoforms are sequentially activated at different stages of muscle development (fetal vs. neonatal). In the second,

genes again of a single type but encoding particular isoforms are activated in different muscle types (e.g., fast vs slow twitch). The myosin heavy chain genes provide examples of both (Mahdavi et al., 1986). There are at least five skeletal MHC genes in the rat that are members of the myosin heavy chain gene family. All are somewhat larger than 20 kb in length and are apparently contiguous on the same chromosome in the order of their developmental expression. Three undergo stage-specific regulation. The embryonic MHC gene is fully active in the 20-day fetus and declines in activity to near zero by day 7 after birth. At day 7 in the newborn, however, the neonatal MHC gene is fully active only to decline in activity at two adult MHC alleles encoding fast twitch MHC type IIA and IIB are turned on in all skeletal muscles except soleus where only the type IIA allele is expressed.

Soleus also provides an example of muscle type-specific regulation. In this muscle, the fast IIA allele is active as is the slow twitch type I allele (Mahdavi et al., 1986). Conversely, the slow type I allele is not active in other skeletal muscle types at any developmental stage except in cardiac muscle from the left and right ventricles, and two muscles that contain slow twitch fibers (extraocular and diaphragm). Thus, muscle groups harboring both fast and slow twitch fibers have activated appropriate fast and slow type alleles.

Another mechanism generating two isoforms occurs at the level of promoter selection in the rat myosin light chain gene (MLC). Here, the gene contains two different promoters separated by over 10 kb of sequence (Nadal-Ginard et al., 1986). Because a new exon occurs in the additional 10 kb of DNA, transcripts from the upstream promoter are spliced differently from those from the downstream promoter yielding the different amino acid sequence in the N-terminus of the two MLC isoforms. Both promoters are active in fast skeletal muscle and are inactive in other muscle types.

How can different promoters be active at different stages of muscle development and in different muscle types? Current studies in many laboratories are directed toward finding specific transcription factors and the specific DNA sequences at which they bind to promote transcription (Muscat, Kedes 1987; Nudel et al., 1986). The many genes and different muscle groups in vertebrates may indicate that there will be a large number of such factors.

Isoform Diversity by Alternative Splicing of Transcripts from Muscle-Specific Genes

Alternative splicing of transcripts arising from a single gene to generate multiple messenger RNAs is another mechanism in muscle by which isoform diversity is generated (Emerson et al., 1986). Alternative splicing is also under muscle type- and stage-specific control. One form of alternative splicing has already been described for the MLC gene where an upstream promoter introduced a new exon which gave rise to changes in the amino acid sequence in the N-terminus. Another pathway gives rise to changes at the C-terminus because transcription terminates upstream from the normal termination site, thereby omitting downstream exons. The tropomyosin gene, for instance, is transcribed from the same promoter in both skeletal and smooth muscle but is terminated "prematurely" in skeletal muscle generating

a truncated messenger RNA (Nadal-Ginard et al., 1986). As a result, the skeletal muscle transcript is spliced differently than the smooth muscle transcript leading to entirely different sequences in the final 26 amino acids of tropomyosin.

Another complex pathway involves splicing of transcripts from the troponin T gene (Emerson et al., 1986). In this case, the gene is transcribed and terminated identically in various muscle cells but exons are joined together by differential splicing in a variety of ways depending on the muscle-type and developmental stage. For example, the rat troponin T₁ gene is over 20 kb in length and contains 18 exons. From the DNA sequence, at least 64 different splicing pathways are possible and 10 have already been detected (Nadal-Ginard et al., 1986).

Needless to say, the regulated expression of contractile protein genes is complex and the details are being worked out by a large number of laboratories. Without knowledge of the structure of the genes, their sequence and their transcription patterns, unravelling these pathways would have been exceedingly difficult, if not impossible.

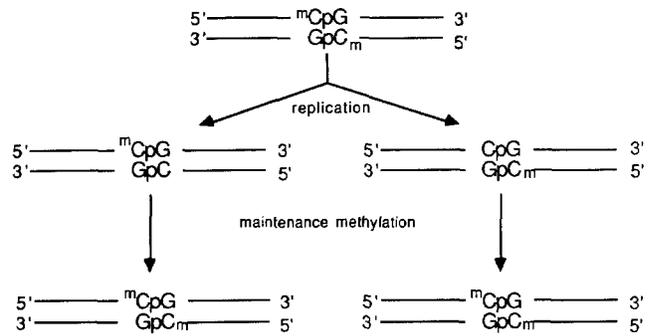
DNA Methylation and Chromatin Structure

Although it was stated in the Introduction that all cells of an organism contain identical information, this refers only to the base sequence of genomic DNA. That is, all cells contain the same amount of DNA and the same DNA sequences. (B and T lymphocytes are an exception.) However, expressed genes differ from unexpressed genes in at least two ways that are important for developmental regulation of tissue-specific genes. As illustrated in Figure 2, the MHC gene is held in an "open" or accessible configuration in chromatin for transcription in myotubes while it is maintained in a "closed" or inaccessible configuration in somatotrophs. Conversely, the GH gene is accessible in somatotrophs but not in myotubes nor in all other tissues of the organism. Accessibility for transcription also applies to housekeeping genes, which are kept accessible in all terminally differentiated cells.

A useful way to think about accessibility is that expressed genes and their DNA regulatory sequences must be available for recognition by RNA polymerase II and other transcription factors. The empirical basis for this idea has come from experiments showing that deoxyribonucleases can digest genes more rapidly in nuclei or chromatin from cells actually expressing the genes than from cells not expressing the genes (Weintraub, 1985). Furthermore, regions flanking the 5' end of expressed genes are "hypersensitive" to digestion with DNases, implying an even more accessible configuration in those areas.

Apart from their inaccessibility, unexpressed genes are also "marked" by methylation of cytosine residues (Razin, Riggs 1980; Doerfler 1983; Cedar 1988). Conversely, expressed genes are usually unmarked because they lack methyls on most of their cytosines. In vertebrates, the only modified base is cytosine and ~1/20th of all cytosines are methylated. Methylation occurs at C5 of the pyrimidine ring and the majority of the 5-methylcytosines occur in the rare dinucleotide 5'-CpG-3'. As shown in Figure 6, both cytosines are symmetrically methylated on each strand of DNA. Replication creates a half-methylated site which is then methylated by a "maintenance" DNA methyltransferase using S-

Figure 6



Maintaining the methylation state of cytosine residues in vertebrate DNA.

adenosyl-L-methionine as a methyl donor. Hence, a methylated CpG site can be maintained in a methylated state and transmitted faithfully to daughter cells. In terms of gene expression, this means that a methylated gene can be held in an inactive state in a cell and all of its descendants.

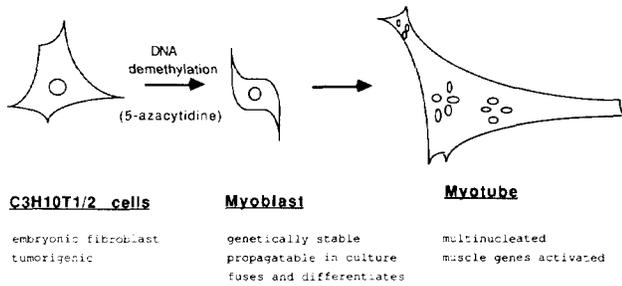
In terms of terminal differentiation, methylation appears to play an important role in gene activation. This is illustrated in Figure 2 where the MHC gene is unmethylated in myotubes and heavily methylated in somatotrophs. Conversely, the GH gene is unmethylated in somatotrophs and methylated in myotubes. It appears, therefore, that demethylation of the muscle-specific set of genes accompanies their activation during terminal differentiation.

An important aspect of methylation with regard to determination revolves around its effects on gene expression and the heritability of the methylation state. For instance, introducing methyls into CpG sites near promoters of some genes *in vitro* blocks the expression of the genes after they are introduced into cells in culture by DNA-mediated transfection (Razin, Riggs 1980; Doerfler, 1983; Cedar, 1988). Furthermore, methylation of a skeletal muscle actin gene not only silences the gene in nonexpressing cell types (Yisraeli et al., 1986), but also prevents the formation of DNase-hypersensitive sites on modified DNA after introduction into cultured cells (Keshet et al., 1986). Because methylation can alter the transcription state of genes and propagate that state to daughter cells, considerable attention has focused on methylation playing a key role in determination. Indeed, as discussed next, demethylation of a single gene in a cultured mesodermal cell line can convert the cell to a myoblast that ultimately fuses to form myotubes.

Mouse Embryonic C3H10t1/2 Cells and the Identification of Two Genes Involved in Myogenic Determination

C3H10T1/2 cells are a tumor cell line (Constantinides et al., 1977) established from embryonic mouse fibroblasts (Figure 7). After treatment with a potent inhibitor of DNA methylation (5-azacytidine), colonies arise among the rare survivors that contain multinucleated myotubes which can be induced to twitch by stimulation with acetylcholine (Constantinides et al., 1977). Myotube-containing colonies arise at an

Figure 7

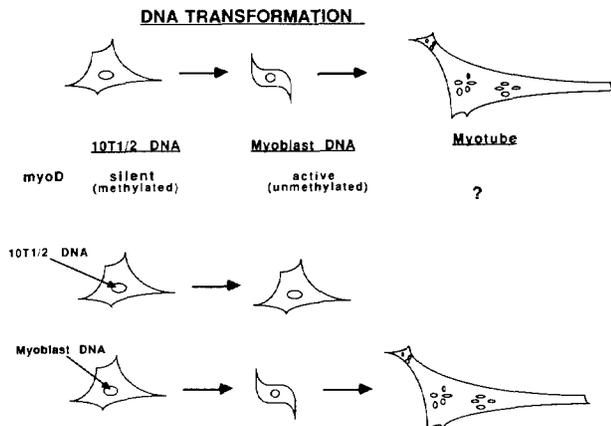


Properties of C3H10T1/2 cells and their conversion to myoblasts that differentiate into myotubes.

astonishingly high frequency (>25%) while colonies containing two other differentiated cell types (adipocytes and chondrocytes) also arise but at much lower frequencies (Taylor, Jones 1979; Konieczny, Emerson, 1985; Konieczny et al., 1986). Importantly, many cells in a myotube-containing colony are not incorporated into the myotube itself, so unfused myoblasts can be isolated and stably propagated. Under appropriate culture conditions, the myoblasts can then be induced to differentiate into myotubes that have activated genes encoding several contractile proteins, e.g., myosin heavy chain, myosin light chains (1emb,1,2,3), α -actin, α , β -tropomyosin, and troponin C and T (Konieczny, Emerson 1984). Genes for these sarcomeric proteins are not expressed in the myoblast lines themselves.

These observations suggested a simple hypothesis, termed the "master gene" hypothesis (Konieczny et al., 1986). The hypothesis and a test of its major prediction are diagrammed in Figure 8. A single gene (i.e., the myogenic determination gene) is activated by demethylation and converts the 10T1/2 cell to a proliferating myoblast that can differentiate into myotubes. A direct test of the hypothesis was undertaken independently by two labs using DNA-mediated transformation of 10T1/2 cells to myoblasts by DNA from myogenic but not nonmyogenic cells. When DNA from

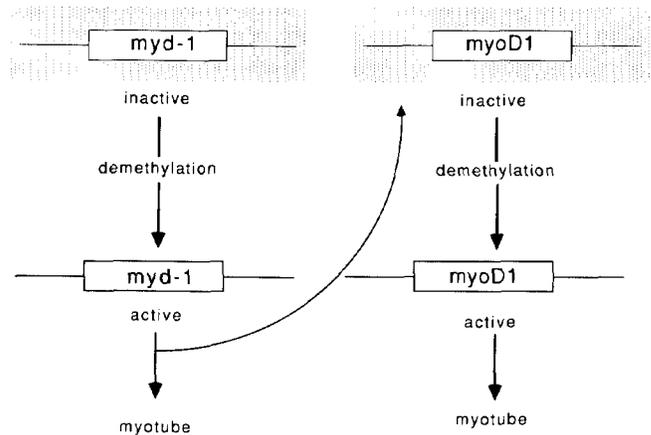
Figure 8



Master gene hypothesis of myogenesis and its test by DNA-mediated transformation of 10T1/2 cells to myoblasts by myogenic DNA.

Figure 9

Myogenic Determination Pathway



Myd1 and myoD1 genes and their activation during determination in mesodermal 10T1/2 cells.

5-azacytidine-induced 10T1/2 myoblasts or other rodent myogenic lines (Lassar et al., 1986) or DNA from quail myoblasts or unmethylated human DNA (Konieczny et al., 1986; Mahdavi et al., 1986) is introduced into 10T1/2 cells, the cells are transformed into myoblasts that subsequently differentiate into myotubes with appropriate muscle gene expression. In control experiments, DNA from parental 10T1/2 cells and other nonmyogenic lines is ineffective in transforming 10T1/2 cells to myoblasts. Given that DNA from widely divergent species (birds to man) functioned identically in the transfection assays, there must be remarkable conservation of genetic function. Two different genes, termed *myoD1* (Pinney et al., 1988) and *myd1* (Lassar et al., 1986) have since been identified, both of which have the following property: When the *myd1* gene or the *myoD1* gene (actually a "chimeric" gene driven by a retroviral promoter) is introduced into 10T1/2 cells, the cells are converted into myoblasts that are capable of myotube formation (Figure 9). The cDNA to *MyoD1* has been cloned and sequenced (Pinney et al., 1988). *Myd1* is still in the process of being isolated.

Virtually nothing is known about how the two genes function. It is clear, however, that neither is a simple transcription factor that activates the set of muscle-specific genes. This was deduced from experiments in which a foreign (nonmurine) myosin or troponin gene was introduced into 10T1/2 cells before conversion to myoblasts by 5-azacytidine (Konieczny, Emerson, 1984; Lassar et al., 1986). The resulting myoblasts did not express the foreign myotube-specific genes until **after** myotube differentiation. The two genes appear to be unrelated in that they do not cross-hybridize on Southern blots. Also, there is a suggestion that *myd1* may be the "master" gene that underwent the actual demethylation event because *myd1*-transformed 10T1/2 cells actively transcribe the *myoD1* gene.

Summary

In broad terms, myogenesis results from a two-stage process in which cells are first determined to form muscle and then differentiate to express a given muscle phenotype. Determination involves a transformation of mesodermal stem cells into proliferating myoblasts which are committed, presumably irreversibly, to the myogenic pathway. Differentiation involves withdrawal of myoblasts from the cell cycle and the elaboration of an organized contractile apparatus, neuromuscular synapses and metabolic enzymes required for muscle function. Commitment of stem cells to the myogenic lineage involves expression of a small number of genes that shunt stem cells into the myogenic pathway. Expression of these "master" or "switch" genes appears to be controlled at the level of DNA methylation. Subsequently, a panoply of

genes encoding structural and enzymatic proteins of neonatal and adult muscle are activated to generate the fully differentiated muscle phenotype. After gene activation, other regulatory events can occur at other levels of gene expression including, at the very least, messenger RNA processing. A complete understanding of the molecular events which underlie muscle development may allow us to gain some measure of control over the events which lead to the formation of muscle in the adult organism.

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Discussion

D. Anderson: If we could control these genes, what do we want to do? Do we want to turn them on or do we want to turn them off?

R. Ivarie: You want to do both to find out what really needs to be done in terms of application. It's hard to know at this point because we know so little about what myoD1 does per se. Hence, we don't know whether it will be a useful target gene for manipulation for meat production. I have a gut feeling that the gene may act as an antigrowth factor because it promotes exit from the cell cycle for the myoblast to terminally differentiate into myotubes. So regulating the level of the gene in terms of its function may be tricky. If you get its expression up too high, you may take the existing embryonic myoblasts and convert them to nondividing muscle quickly. And end up with a "low-muscled" animal. So the trick may be to keep the expression of myoD1 low in the beginning and then release the block later. This also assumes that external agents (hormones, mitogenic factors, or whatever) actually control the gene's activity once it's activated. A second question is whether you can promote specific demethylation of the myoD1 allele, an unlikely possibility at this point.

M. Hunt: Some of us, in some of the review articles that we have read in this area, have heard of some of the things that you call "quantal mitosis" and "proliferative mitosis." Could you interrelate these with this master gene hypothesis? And the second question is: What role does collagen development of types play relative to myofibrillar protein gene turning on? The relationships between some collagenic events and myofibrillar events in the development of the muscle.

Ivarie: With regard to quantal divisions, do I really have to say something? Most of you have been teaching it for a long time and I think the value of the concept as advocated by Holtzer was to point out the power of the determination decision. That's to say that it emphasized that determinative events are transmitted genetically to donor cells in the course of elaboration of cell type lineages. Whether it is truly quantal is, I think, anybody's guess. Quinn and Namerof in Seattle, both former colleagues of Holtzer, have strong data showing that some myoblasts undergo a determinative event resembling a quantal division which is transmitted to daughter cells prior to biochemical differentiation into myotubes.

A property of myoblasts is that they have to maintain a proliferative state over a protracted period of animal growth and yet by the time adulthood is reached, myoblast proliferation has to slow down and eventually stop. During proliferation, of course, a fraction of myoblasts continually fuse into myotubes and terminally differentiate.

Quinn and Namerof have found that there is a "quantal" division that takes place at least four divisions before overt biochemical differentiation itself occurs. The experiments are fairly complicated but were done by analyzing individual clones of myoblasts from embryonic muscle in culture and

scoring the number of cells in each clone that biochemically differentiated at various times.

Stockdale and his colleagues at Stanford have undertaken similar experiments and identified clones that contain cells that express only fast or slow twitch myosin which will eventually give rise to fast or slow twitch myofibers. Again, determinative events must have taken in those clones prior to cell culture that was transmitted to their descendants faithfully.

Although I talked to the question, I didn't really answer it. We are now in a position with myoD1 to put the gene under our control and ask those kinds of questions in a very explicit way. What was your second question?

Hunt: Would you comment if there is any relationship between collagen development and myotube development?

Ivarie: Yes. They both come out of the mesodermal lineage. Collagen is expressed by chondrocytes. These 10T1/2 cells also give rise to two other differentiated cell types: adipocytes and chondrocytes, albeit at lower frequency than myoblasts. So in that sense, 10T1/2 cells reflect an early set of events capable of giving rise to three different cell lineages and cartilage is one of those. Whether there is a direct effect in which collagen can act directly on myoblast development, I don't know. Kauffman in Illinois has some evidence on that. If anybody can address this aspect, they should come to the microphone.

D. Beermann: Dr. Ivarie, we seem to be willing to accept the postnatal growth explanation for stimulation of mitosis being related to the elevation of growth hormone, that being tied to elevation of IGF1 and now we see developing a fairly detailed story about how there is both autocrine and/or paracrine involvement in the regulation of IGF1 expression within muscle which opens up new thoughts about how the gene expression for myofibrillar proteins occur postnatally. What I'm asking is: Do you have any ideas about whether such a growth factor or small peptide hormone which would be present in the circulation or maybe be produced in a stemline of cells that are as of yet undifferentiated might be so specialized to be involved in the control of these master genes (MyoD1 or myd1)? Where is the control of these master genes? Is it possible that it's at that general physiological level within a tissue or is it something that's totally unidentified at this point?

Ivarie: The question concerns, I think, what steps occur before mesoderm and what promotes activation of the gene(s) itself. As far as I know, there is virtually no evidence on either question other than to say that we know that once the myoD1 allele is demethylated, it becomes fully active. Once active, it promotes biochemical differentiation into myotubes. Whether it's the demethylation event itself that activates the gene or whether it's the consequence of activation by other kinds of factors is a completely open question, but a very good question.