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Gene Regulation and Developmental Patterns of Muscle Differentiation

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Differentiation of the skeletal muscle tissue (myogenesis) has been subject to considerable attention over the last decades with particular emphasis on the gene regulatory aspects. In the present review we are attempting to survey our present knowledge regarding the stepwise genetic control operating from the early stages of somitogenesis and somite patterning til the formation of adult muscles. The modes of action of myogenic regulatory genes (MyoD family, MEF_2) are discussed at the light of recent data concerning the influences of chromatin remodeling, of the cell cycle, and of the various signalling factors derived from the non-muscle tissues neighbouring the paraxial mesoderm.

Key Words: Paraxial mesoderm, Somites, Signalling molecules, B-HLH factors, Cell cycle, Histones, Myf5 promoter

The present review is devoted to some recent data illustrating the genetic control of tissue-specific development, using the skeletal muscle as a model. Skeletal muscle cells constitute one of the most powerful model system for uncovering the molecular mechanisms of cell fate specification (Buckingham 1985, 2001). Moreover the gene regulatory cascade involved in the control of myogenesis has recently been enriched by the discovery of many new intermediates in addition to the early characterization and analysis of myogenic regulatory genes from the MyoD family. In particular the role of myogenic regulatory genes in chromatin remodeling has been explored and people have gained more insights into the relation between the cell cycle exit phenomenon and the onset of muscle differentiation. Finally, and more importantly, a clearer picture has recently emerged concerning the in vitro spatiotemporal interactions - mediated by signalling molecules - between embryonic precursors of muscle cells and neighbouring non-muscle tissues, prior to the acquisition of the myogenic phenotype.

Early Embryogenesis and Main Developemental Steps

In vertebrates, (the mouse being used here as a model), the skeletal muscle tissue originates from the so-called «third embryonic layer», the mesoderm, a territory of the developing embryo from which derive other tissues (ex: bones, cartilage of the ribs and dermis) (figure 1).

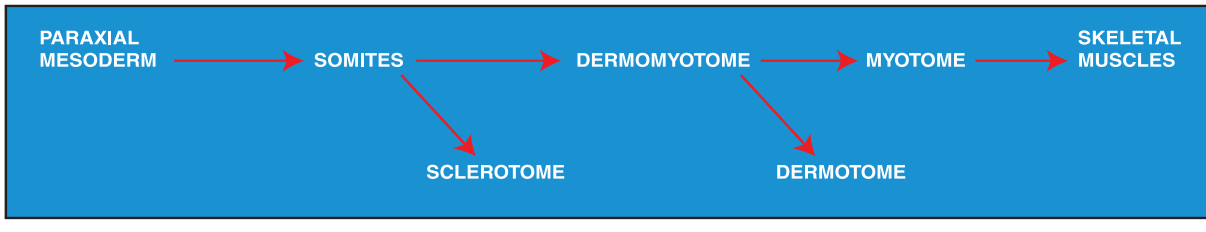

Figure 1. Schematic Developmental Cascade leading to skeletal myogenesis

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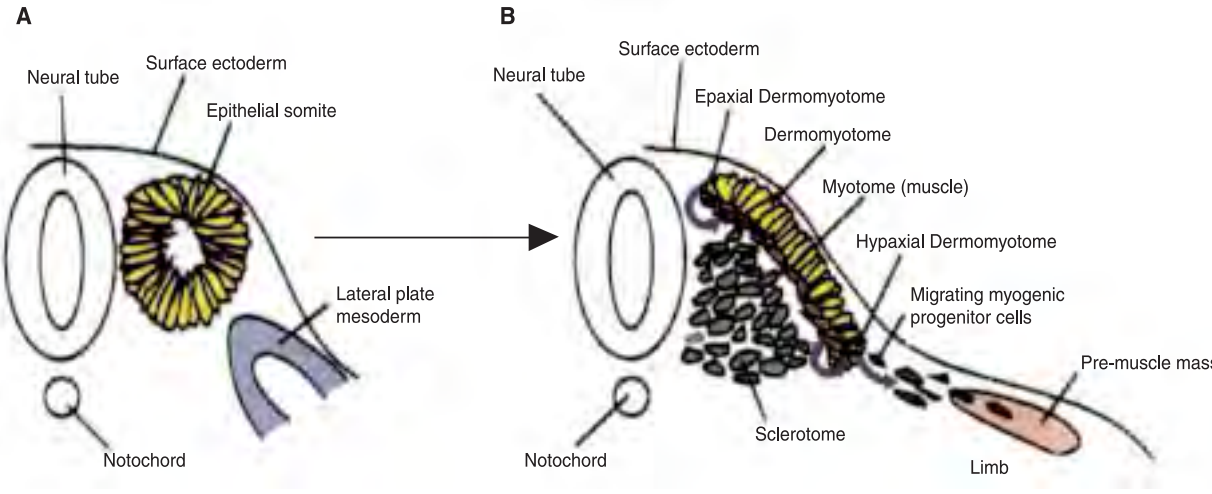

Figure 2. Patterning of differentiating somites, roles of neighbouring tissues, and early myogenic events. Reproduced from F. Relaix, M Buckingham, Genes and Dev (1999), **13** 3171]

Myogenesis more specifically proceeds from the paraxial mesoderm, an undifferentiated tissue located in close vicinity to axial elements of the body such as the neural tube and the notochord. Before the appearance of the first muscle progenitor cells, the paraxial mesoderm becomes re-organized into a series of round-shaped epithelioid cell masses, called «somites», whose disposition runs parallel to the body vertical axis (spine, neural tube). Complex rearrangements (patterning) are next taking place inside each individual somite according to a rostro-caudal (head to tail) gradient; they involve many cell migratory and stepwise differentiation events whose occurrence is depending upon signalling factors emitted by the neighbouring tissues (neural tube, notochord, lateral plate mesoderm, surface ectoderm) (figure 2). These developmental stages comprise the formation of cellular territories called: sclerotome (at the origin of ribs, cartilage, etc.) dermomyotome (DMM) later subdividable into the dermatome and the myotome from which derive most of the trunk muscles. Limb muscles, as well as the diaphragm and the tongue, originate from a population of progenitor cells which migrate away from the DMM. All these embryogenic steps are strictly controlled at the genetic level, both at early stages of somitogenesis and during the patterning of the somites into the various subterritories mentionned above (figure 3).

Moreover these developmental changes, as already stated, are also under the overarching command of diffusible factors secreted by the neighbouring tissues. These signalling effectors are encoded mainly by special members of the following gene families; sonic hedgehog $(Sh)_s$, BMP_s (Bone morphogenetic proteins) and Wnt_s. Sh factors originate from the notochord, Wnt-1 from the dorsal portion of the neural tube, Wnt-7a from the surface ectoderm while BMP4, which transiently represses myogenesis, derives from the lateral mesoderm (Marcelle et al. 1997, Borycki 2000, Tajbakhsh & Buckingham 2000, Wagner et al. 2000).

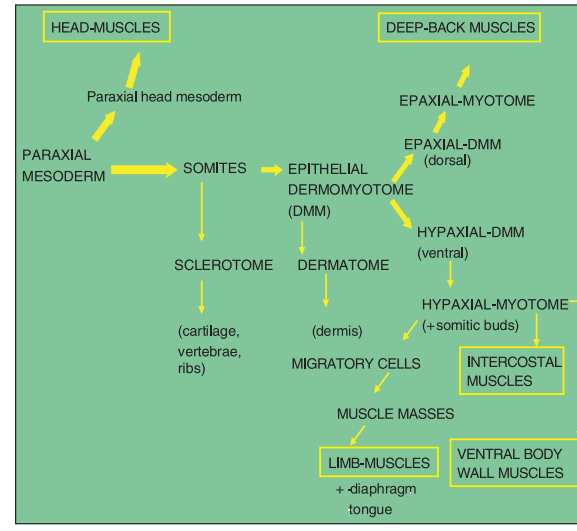

Figure 3. Formation of various muscle compartments in vertebrates

These various signalling factors behave like inducers or repressors of the myogenic program, or else simply maintain its progression or inhibit apoptosis. Overall, they trigger the conversion of undifferentiated cells from the mesoderm into direct progenitors of the muscle tissue.

Terminal myogenesis itself is characterized by the first appearance of mononucleate, actively dividing Myoblasts (Mb). Their division is a prerequisite to the formation of large muscle masses in different parts of the body. Myoblasts then become post-mitotic and beginn to express groups of genes characteristic of the typical muscle phenotype (contractile proteins, enzymes, post-synaptic receptors, etc.); they simultaneously undergo a phenomenon of cytoplasmic fusion to form multinucleate scyncithia, called Myotubes (Mt) which gradually mature into Myofibers (Mf) and finally into adult muscles (figure 4)

Myogenic Regulatory Genes

The developmental cascade involved in the pathway going from muscle precursor cells to adult myofibers is under the control of a family of genes coding for so called «Myogenic regulatory factors» (MRFs). These MRFs are transcription factors which belong to the category of «basic-helix-loop-helix» proteins (b-HLH) and have the capacity to activate «muscle specific» genes containing a special cis-regulatory sequence, the E-box. In vertebrates, 4 MRFs have been reported to exist: Myo D, Myogenin, Myf 5 and Myf 6 (alias MRF4/herculin (Davis et al. 1987, Braun 1989, Edmonson 1989, Wright 1989, Braun 1991) (table I).

Activation of muscle gene expression by myogenic b-HLH proteins is dependent on their association with members of the MEF_2 family, a particular class of transcription factors which bind AT-rich sequences in muscle gene control regions (Black & Olson 1998) and which behave like essential potentiating agents.

Table 1. *Genes encoding regulatory factors in vertebrates*

MYOD	(H. WEINTRAUB, A. LASSAR...)
MYF-5	(T. Braun et al.)
myogenin	(W. Wright; E. Olson)
MRF-4 (alias MYF-6/HERCULIN)	(T. Braun et al.)

MRF's : contain a << B-H-L-H>> motif which binds a cis-acting regulatory sequence (E-box) on <<muscle>> genes (after hetero-dimerisation with factor E-12)

In addition, various studies, initiated by Gerber et al. (1997) have revealed a crucial role of histone modifying enzymes: histone acetyl transferases (HATs) and histone deactylases ($HDAC_6$), in regulating activation and repression of the differentiation program respectively (McKinsey et al. 2001).

In their deacetylated form, histones lead to the packaging of DNA around nucleosomes (condensed chromatin) a conformation of chromatin that prevents access of transcriptional activation factors to their target sites. By contrast histone acetylation results in a relaxation of the nucleosomal structures allowing access of these factors to their sites. Among the histone transacetylases, attention was more specially focused on pCAF and p300 (Sartorelli et al. 1999, Polesskaya et al. 2000) which can associate with Myo D on E-box elements. Disruption of complex formation between p300 and Myo D, resulting from the action of an anti-p300

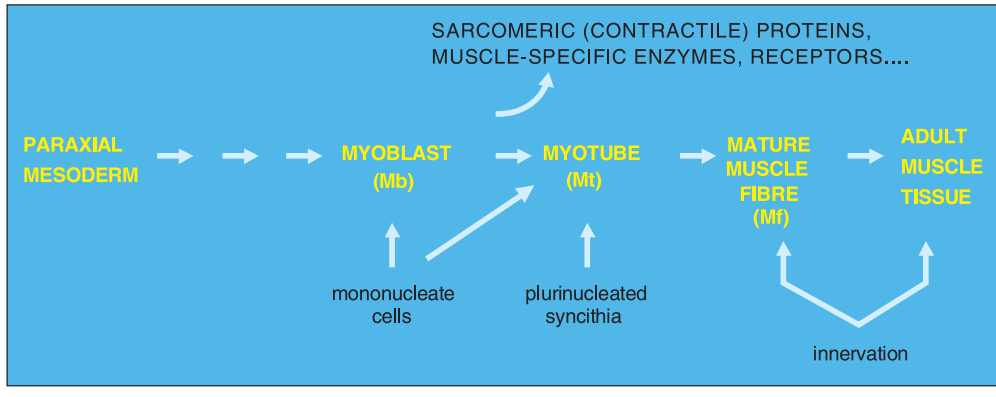

Figure 4. Ontogenic Transitions during myogenesis

antibody causes repression of Myo D mediated transcription (Puri et al. 1997a). Yet p300 HAT activity was found dispensable for activation of Myo D and muscle differentation and it is believed that p300 regulates Myo D by serving as a bridge to recruit PCAF whose role appears more critical (Puri et al. 1997b).

Myogenic Regulatory Factors and Cell Cycle

Many studies have been devoted to the cessation of division as a prerequisite to terminal differentation following the early observations by Stockdale and Holtzer (Dienstman 1977). For recent reviews see (Sabourin & Rudnicki 2000, Zhu & Skoultchi 2001).

Not only have people analyzed the fate of myogenic factors during the cell cycle of actively dividing myoblasts, but they have also attempted to understand why these factors are unable to induce differentiation as long as cell division proceeds in growth factor rich media, what mechanisms cause the arrest of this division and what is the nature of the switch from the dividing to the differentiating stage.

Myf 5 and Myo D are specifically degraded at mitosis and at the G1/S phase respectively. This degradation involves phosphorylation by cyclin-dependent kinases $(CDK)_s$ (Lindon 2000). Myf 5 but not Myo D, is high at Go (Kitzmann & Fernandez 2001). If one prevents Myo D degra-dation either by blocking the G1/S specific cdk, by some interaction with p57, a cdk inhibitor, or with pRb (an E2 F inhibitor), this causes cell cycle arrest and differentiation (Reynaud et al. 2000).

Many mechanisms have been proposed with the view of explaining why Myo D cannot cause differentiation as long as myoblasts keep dividing. It has been proposed for instance that cdk-dependent phosphorylation of Myo D might prevent it from being translocated into the nucleus. Alternatively, the role of Id, a HLH protein lacking a basic domain but capable of forming a transcriptionally inactive heterodimer with E_{12}, and the synthesis of which is stimulated in mitogen-rich media, has also been invoked (Benezra et al. 1999). The expression of histone deocetylases that bind MEF_2 and Myo D in myoblasts could also provide a potential explanation for the paradoxical observation that these transcription factors while present in myoblasts remain inactive (Lu et al. 2000). As to the switch from division to differentiation, it is well known that withdrawal of growth factors from a rich medium supporting myoblastic growth triggers a coordinaed process of cell-cycle exit and myogenic differentiation. Forced expression of cdk inhibitors (CKIs) such as $p21^{C1P1}$ and $p16^{INK4a}$ suffices to cause cell cycle withdrawal and expression of certain differentiation markers (Shapek et al. 1995). Similar effects have been observed when overexpressing other CKIs such as $P57^{KIP2}$ and $p27^{CIP1}$. By contrast, overexpression of cyclin D1 inhibits Myo D activity.

While it is now clear that cdks can negatively regulate the function of myogenic transcription factors, a symmetrical situation is also observed. Myf 5 and Myo D can induce cell cycle arrest in the absence of myogenic differentiation (Montarras et al. 2000). As far as Myo D this effect is probably due to its capacity to transcriptionnaly activate the CKI inhibitor $p21^{C1P1}$ independently of p53 (Guo et al. 1995) and, possibly also to its direct physical interaction with cdk4. In conclusion, recent studies have revealed that the coordination of cell proliferation and differentiation is achieved through the dual function of the regulators involved in each of these processes: for example certain cell cycle regulators, such as CKI's can induce differentation while certain differentiation-promoting factors (ex: Myo D) can directly regulate proliferation.

Specific roles of Myogenic Regulatory Factors during Differentiation

Many experimental approaches, both *in vitro* and *in vivo*, have been used to characterize the particular stage at which each member of the Myo D family is involved. Homologous recombination experiments designed to invalidate these genes, either singly or in combination (Olson et al. 1996, Kaul et al. 2000) have indicated that Myf 5 and Myo D are expressed in the paraxial mesoderm and are involved at the early stages of myogenic determination of the precursor cells. By contrast, Myogenin, MRF 4 (but also Myo D) play a major role in controlling later stages, for example the conversion of myoblasts into myotubes, and the

expression of late genes in the adult muscle. In the absence of Myogenin, Myo D and MRF 4, muscle differentiation does not occur both *in vivo* or *in vitro*, showing that Myf 5 alone cannot activtate differentiation (Valdez et al. 2000).

Signalling Systems and Control of Myogenic Regulatory Genes

Recent studies have shed light on the mechanisms whereby signalling molecules secreted by neural tube, ectoderm, notochord, etc.. ultimately induce expression of MRF genes which, in turn, control terminal myogenesis. In addition to genes encoding effectors from the Sh, BMP and Wnt families already mentionned (see above), another category of genes was found to be involved in mediating local instructions to the target MRF genes. Pax-3 (Goulding et al. 1991), Six-1 (Oliver et al. 1995), Eya-2 (Xu et al. 1997), Mox1/2 (Candia et al. 1992), Gli 2/3 (Hui et al. 1994) as well as Sni/Sno (Relaix & Buckingham 1999) and LBx-1 (Brohmann et al. 2000, Gross 2000) have recently received a particular attention (figure 5). Pax-3 (Bober et al. 1994, Ridgeway & Skerjanc 2001), a member of the paired-box family of transcription factors is probably involved at the beginning of the myogenic program by activating Myo D Its expression is detected in the paraxial mesoderm and in the entire somite while it is later confined to the developing DMM and the maturing cells of the dorsomedial lip. In Pax-3 $^{-/-}$ mice, cell migration giving rise to hypaxial myogenesis does no longer take place. Pax-7, a closely related member of the pair-boxed genes family is playing a similar role as Pax-3 and can often compensate for its defect. Yet, according to recent study, it would be more specifically involved in the specification of satellite cells (Seale et al. 2000). Accordingly, in Pax-7 null mice, muscle satellite cells are absent. Pax-3 transcripts are not detected in satellite cells, the differentiation of which requires Myo-D. Table 2 summs up the major properties, as well as mechanisms and sites of action for the major signal mediating genes: Six-1, Eya-2, Mox-1 and-2, and Gli 2/3 as well as dach-2, Ski and Sno.

Of particular interest, from the stand point of molecular evolution, is the conservation of genes Pax, Six, Eya and Dach for they have been characterized in both invertebrates (such as *Drosophila*), and in all types of vertebrates. Quite remarkably however, and as discussed by Heanue et al (Heanue et al. 1999) as well as by F Relaix and M. Buckingham (Relaix & Buckingham 1999), these genes were found to be involved in different developmental pathways. For example, the same combination of transcriptional regulators required for eye formation in *Drosophila* has been redeployed elsewhere during vertebrate embryogenesis, for example, in the somite and its skeletal muscle derivatives. This situation is in accordance with the well known «tinker» hypothesis first proposed by François Jacob some years ago (Jacob 1981)

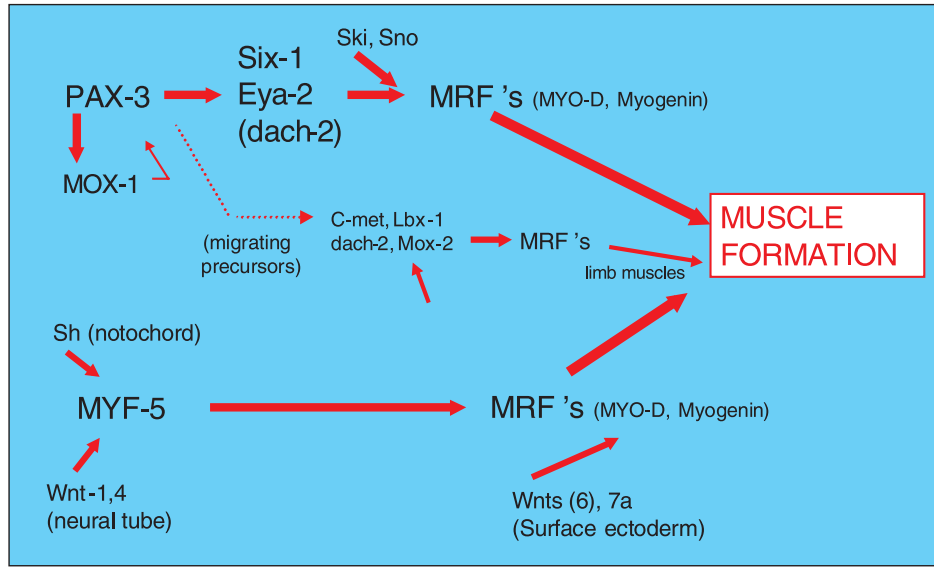

Figure 5. Par-3 and Mys-5 Dependent Myogenic Pathways

according to which evolution rather proceeds by adopting new combinatorials of ancient genes, to serve new physiological functions rather than by making use of new genes.

Figure 5 attempts to summarize the stepwise cascade of regulatory gene expression taking place prior to, or following the stage of myogenic determination. As can be seen there exist, at least, two possible regulatory «routes» where to engage mesodermal, undifferentiated cells into skeletal myogenesis:one is Myf-5 dependent, and the other Pax-3 dependent (Tajbakhsh et al. 1997).

The exact significance of this phenomenom is not clear. Either this would reflect some heterogeneity among the muscle precursor cells, or the two pathways involved for entering the myogenic lineage would differ, depending upon some kind of threshold imposed upon by the signalling factors that are secreted by the mesoderm neighbouring tissues. Whatever it is, Pax-3 is sufficient to drive skeletal myogenesis when overexpressed in a pluripotent cell line, and, from the stand point of the temporal expression pattern, it presumably induces expression of Six-1 and Eya-2 prior to the expression of MyoD and myogenin (Ridgeway & Skerjanc 2001).

Myf-5 playing also a key role in early muscle cell determination in response to signalling cascades, recent work (Hadchouel et al. 2000, Summerbell et al. 2000) has been devoted to analyze in details the organization of the Myf-5 promoter region with the aim of understanding how this promoter might respond and interact with the signalling factors. Using a YAC* transgenic approach, Buckingham's group has undertaken to identify the regulatory «domains» present in the Myf-5 promoter gene that control the spatiotemporal expression of this gene during myogenesis in the mouse embryos. They have found that the Myf-5 promoter can be «dissected» into discrete «modular regions» over 96 Kb upstream of the Myf-5 gene, each of the characterized «module» being responsible for the expression of Myf 5 within very defined and restricted «types» of muscles, and at precise stages of their development. It may be that each modular segment of the Myf-5 promoter does bind a special (regulatory) protein, that would be encoded by «signalling genes» (such as Sh, Wnt's, BMP's, etc.), and that the close interactions of these proteins, when bound *in situ*, would elicit variable three dimensional conformations of the Myf-5 promoter, causing a finely-tuned expression of the corresponding gene.

Table 2. *Genes involved in signalling events and myogenesis*

Genes	Category	Expression and Effects
PAX- 3	pair-boxed	-Paraxial Mesoderm, stimul. MYOD
SIX-1	transcript. factor	-stimulates myogenin
Eya-2, Dach-2	interact with SIX-1	-dach-2 expressed in epaxial DMM premuscle masses of limb buds, lateral plate mesoderm....
MOX-1, MOX-2 (DMM)	homeobox-genes	-Expression of MOX-1 in paraxial mes. MOX-2 migrating precursors
Gli-1,2,3	« Zn finger» types	-transcript. factors, transduction of Sh effect
Ski	component of histone deacetylase	-stimulates myogenin
Sno	onco-protein	- «id »
C-met	encodes tyrosine kinase receptor	-delamination in epithelioïd somites
Lbx-1	homeobox-gene	-in migrating muscle progenitor cells
Sh	signalling factor	-released from notocord, stimulates Myf-5
Wnt-1,7	signalling factor	-Wnt-1 (neural tube) stimulates Myf-5 Wnt-7 (from surface ectoderm) stimulates MYOD

Conclusion

A complex but very precise picture of how genes and the corresponding factors take part in controlling the formation of various vertebrate body muscles is presently available.

Many new insights have been gained into the way locally secreted signalling molecules derived from non-muscle neighbouring tissues and muscle specific regulatory genes do interact to tigger the conversion of mesodermal precursors into differentiated skeletal muscles. As to the molecular mechanisms underlying the function of myogenic regulatory genes themselves, great progress was made in our understanding of chromatin remodeling accompanying their activation of target

* This refers to a gene or c-DNA "shuttle vector" which can function in both *E. coli* and yeast. It is in fact a yeast minichromosome which can accomodate large DNA fragments (several hundreds kilobases long) for cloning.

genes. The well-known inverse relationship between cell proliferation and differentiation during myogenesis has also been explored in great details. Recent data support the idea that it may result from dual functions residing in the central regulators of proliferation allowing them to also regulate differentiation.

Yet, many unknowns subsist, particularly in the spatio-temporal integration of these various developmental events during *in vivo* myogenesis. The increasing number of genes and intermediates involved will probably call for simulatory models that could help addressing their complex interactions. Use of microarray techniques might also shed light on the coordinated expression of the regulatory genes at particular phases of development. Moreover three dimensional representation of promoter-bound associations of regulatory proteins should constitute an important direction for future research.

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