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Review

Control of skeletal muscle-specific transcription: involvement of paired homeodomain and MADS domain transcription factors

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Introduction

Analysis of skeletal muscle development provides a successful approach to deciphering the molecular mechanisms which control gene expression during cell differentiation (Buckingham, 1992; Olson, 1992). Figure 1 summarizes the first step toward the formation of skeletal muscle, which occurs when mesodermal progenitor cells, mostly located in the somitic mesoderm, become committed to forming myoblasts, which are then restricted to a myogenic fate (Fig. 1) (Buckingham, 1992; Olson, 1992). These determined myogenic cells grow actively and do not differentiate until growth stimulation stops, leading to their exit from the cell cycle and their fusion, allowing them to form multinucleate myotubes which start to express a new set of genes encoding differentiation markers (Christ *et al.*, 1977; Buckingham, 1992; Olson, 1992; Ordahl and Le Douarin 1992; Sassoon, 1993). These determination and differentiation steps have been extensively studied *in vitro*, using the multipotential mesodermal C3H/10T1/2 cell line and a variety of myoblast cell lines (Buckingham, 1992; Olson, 1992). The results obtained with these *in vitro* systems are schematized in Figure 1. *In vivo*, skeletal myogenic progenitor cells originate mostly from the somitic mesoderm and to a lesser extent from the prechordal plate which will give rise to some head muscle, while cardiac and smooth muscle cells have a different embryological origin (Hogan *et al.*, 1985; Christ *et al.*, 1986; Buckingham, 1992). Somites arise from the segmentation of the paraxial mesoderm around day 8 of development in the mouse, starting from the anterior part of the embryo toward its caudal end (Hogan *et al.*, 1985; Christ *et al.*, 1986). As soon as they are formed, they differentiate into sclerotome, contributing mostly to the axial skeleton, and dermomyotome which will then give rise to the dermatome and the myotome (Hogan *et al.*, 1985; Christ *et al.*, 1986). The dermatome will contribute mainly to dermal structures while the myotome will give rise to the major part of the skeletal muscles of the animal (Hogan *et al.*, 1985; Christ *et al.*, 1986; Buckingham, 1992). In contrast to trunk skeletal muscles, limb skeletal muscles do not derive from the myotome but from precursor cells that migrate from the ventrolateral dermomyotome of early somites (Christ *et al.*, 1986; Ordahl and Le Douarin, 1992; Sassoon, 1993). These migrating cells do not express known markers for skeletal muscle. Once they have reached the limb, they differentiate into muscle only after several days.

In the last few years, decisive progress has been made towards understanding the molecular mechanisms involved in the control of skeletal myogenesis, mostly due to the use of molecular genetics (Buckingham, 1992; Olson, 1992). Indeed, a large number of genes encoding skeletal muscle differentiation markers have been isolated and their regulation studied in great detail (Buckingham, 1992; Olson, 1992). While most of these genes are only expressed in fully differentiated myogenic cells (myotubes), only a small number of genes (desmin, ß-enolase) start to be expressed in proliferating myogenic precursor cells (myoblasts) and remain expressed in the differentiated myotubes resulting from myoblasts fusion (for references, see Li *et al.*, 1993).

More recently several myogenic control genes have been isolated and found to constitute a small gene family encoding myogenic helix-loop-helix (HLH) transcription factors (Lassar *et al.*, 1987; Buckingham, 1992; Olson, 1992). While the discovery of this class of genes has greatly enhanced our understanding of skeletal myogenesis, there is a growing body of evidence to suggest that other types of positive regulators of the myogenic process must act in cooperation with the aforementioned HLH control genes to trigger skeletal myogenic determination and differentiation. In this review, we will not discuss cellular and embryological aspects of myogenesis, which can be found elsewhere (Hogan *et al.*, 1985; Christ *et al.*, 1986; Buckingham, 1992; Ordahl and Le Douarin 1992; Sassoon, 1993). We will summarize our current knowledge on myogenic HLH regulators and the relationships of cell proliferation and myogenesis, which have been extensively reviewed recently (Olson, 1992). Only the latest data on the myogenic HLH factors will be developed. We will focus our attention on other classes of transcriptional regulators with emphasis on the involvement of newly described paired homeodomain proteins in the control of muscle-specific gene transcription. Finally, we will discuss the possible functional role of two members of the Pax family of paired domain containing developmental control transcription factors in the control of muscle-specific genes.

The MyoD family of transcription factors

A family of skeletal myogenic Helix-Loop-Helix (HLH) transcription factors has recently been characterized. These factors (Myf-5, myogenin, MyoD and Myf-6), when overexpressed, are able to convert fibroblastic cells of the C3H/10T1/2 line into myogenic precursor cells which can then undergo terminal differentiation (Lassar *et al.*, 1987; Buckingham, 1992; Olson, 1992). It was shown that these factors were able to activate transcription of myotube-specific genes by interacting with common specific target sequences, the so-called E-boxes (CANNTG) (Buckingham, 1992; Olson, 1992). In most muscle-specific genes control regions, two E-boxes located in the same area mediate the effects of myogenic HLH factors. On the other hand, expression of myogenic HLH factors in transfected cells was found to modulate the expression of the endogenous myogenic HLH genes, suggesting the existence of auto and cross regulatory interactions. (Buckingham, 1992; Olson, 1992). In the case of the *myogenin* gene, it was clearly shown that an E-box located in the promoter plays a key role in the regulation of this gene (Chang-Cheng *et al.*, 1993; Yee and Rigby, 1993). *In vitro* experiments using forced overexpression do not allow a clear definition of the respective functions of these regula-

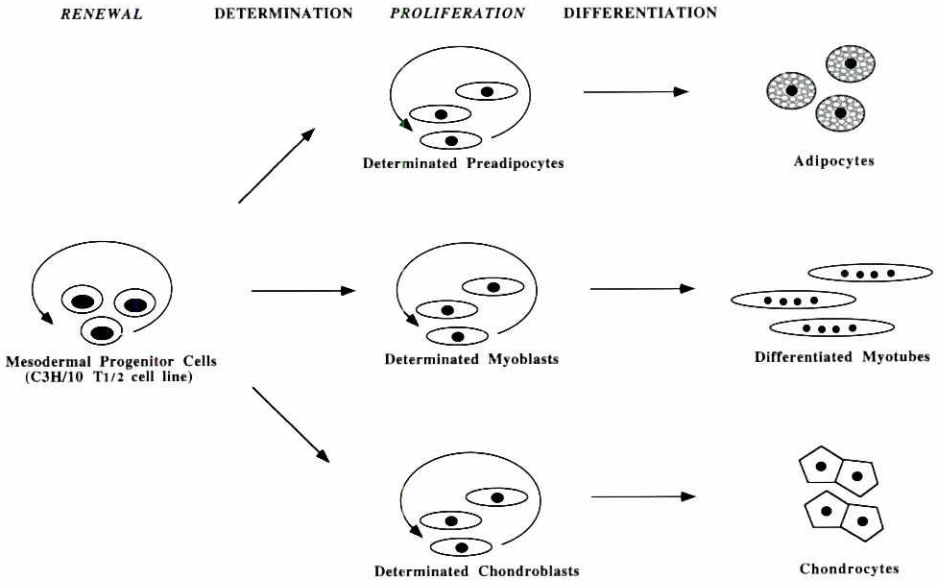

Fig. 1. Schematic representation of the steps involved in the determination and differentiation of the myogenic lineage. *Mesodermal progenitor cells such as C3H/10T 1/2 cells become committed to form determined precursors of the various mesodermal lineages such as myoblasts, adipoblasts and chondroblasts which are restricted in their fate. Upon cessation of proliferation of these determined precursors, differentiation occurs leading to the appearance of differentiated cells characteristic of each lineage.*

tors which can all induce myogenic conversion of multipotential mesodermal C3H/10T1/2 cells. *In vivo* expression data show that the genes encoding these factors are sequentially expressed during myogenesis in the mouse embryo, with *Myf-5* mRNA appearing first at day 8 in the somite and later on in the dermomyotome and myotome, followed by *myogenin*, *Myf-6* and *MyoD* transcripts (Table 1) (Buckingham, 1992). In the limb buds, *Myf-5* mRNA can be detected at day 10.5 and is followed by the expression of the other genes encoding the myogenic HLH factors, which occurs in the same temporal order. Strikingly, no expression of these factors can be detected in the myogenic precursors migrating from the dermomyotome. The existence of committed myogenic precursors in newly formed limb buds can be clearly demonstrated in explant cultures. These explants do not initially express the myogenic HLH factor genes whose expression can be detected after several days of culture (Chang-Cheng *et al.*, 1992; Bober *et al.*, 1994; Tajbakhsh and Buckingham, 1994). Such experiments clearly demonstrate the occurrence of determined limb myogenic precursor cells prior to the expression of myogenic HLH factors and suggest the existence of early determination control mechanisms which do not require the presence of myogenic HLH factors.

The reason why some myogenic HLH regulators such as *Myf-5* are expressed earlier than most muscle differentiation markers, which start to be expressed at day 10.5 *in vivo*, is not clear (Braun *et al.*, 1992; Buckingham, 1992; Olson, 1992; Rudnicki *et al.*, 1992). This is also true for the *in vitro* situation in myoblast cell lines. While several mouse cell lines express high level of *MyoD* mRNA and protein in the absence of expression of muscle differentiation markers, other cell lines do not express *MyoD* transcripts and are characterized by a higher *Myf-5* expression (Montarras *et al.*, 1991; Buckingham, 1992; Olson, 1992). In all cases *myogenin* transcripts appear only when cells start to differentiate, suggesting that this gene is involved in the control of myogenic cell differentiation (Montarras *et al.*, 1991; Buckingham, 1992; Olson, 1992). As *myogenin* gene transcription is controlled through an E-box, it is likely that the Myf-5 protein is involved in the initial activation of the *myogenin* promoter which occurs as early as day 8.5 in the somites. This raises the possibility that the *myogenin* promoter, unlike most muscle-specific promoters, is highly responsive to low amounts of Myf-5 protein. Subsequently, the myogenin gene product might be involved in the activation of muscle specific genes through their E-boxes.

Negative regulation of myogenic HLH factors in proliferating myoblasts

The weak ability of the myogenic factors to activate muscle-specific transcription in myoblasts can be explained at least in part by a variety of inhibitory mechanisms which antagonize the effects of the myogenic HLH regulators by interfering with either the expression or the function of the HLH regulators (Olson, 1992).

One of these negative control mechanisms exploits the fact that, in order to bind to their target sequences with a high affinity and activate transcription of their target genes, myogenic HLH proteins must form heterodimers with the ubiquitous E12, E47 and HEB HLH factors. Indeed the myogenic HLH proteins have a weak ability to form homodimers (Buckingham, 1992; Olson, 1992). Terminal myogenic differentiation requires the inhibition of myoblast proliferation by myogenic HLH factors. This could be at least in part explained by the formation of complexes with the retinoblastoma protein which were recently found in the case of the MyoD factor, and shown to be required for its effects on cell proliferation and differentiation (Gu *et al.*, 1993).

In proliferating myoblasts, a number of proteins interact with the myogenic HLH factors and thus prevent the formation of the aforementioned active complexes (Olson, 1992).The inhibitory HLH protein Id, which lacks the basic region required for DNA-binding, and the c-jun proto-oncogene product form inactive heterodimers with the myogenic factors, preventing their binding to DNA (Olson, 1992). These inhibitory factors appear to be linked to cell proliferation, which antagonizes myogenic differentiation. Indeed, other growth factor (bFGF, TGF-ß) and various oncogenic proteins such as activated fos, ras and src have been shown to counteract the effect of myogenic HLH factors. They act either at

TABLE 1

COMPARISON OF THE EXPRESSION OF PAIRED HOMEOBOX GENES *(MHox, Pax-3* and *7),* MYOGENIC HLH FACTORS AND DESMIN IN EMBRYONIC MOUSE SOMITES AND SKELETAL MUSCLES OF THE TRUNK

Days P.C.	8	8.5	9	9.5	10.5	11.5	12.5	14.5	15.5
Desmin (a)			+	++	++	+++	+++	+++	+++
Myf-5 (b)	+	+	+	++	++	++	+	+/-	-
Myf-6MRF4 (b)	-	-	+	+	+	-	-	-	+
Myogenin (b)	-	*(+)	*(++)	*(+++)	+++	+++	+++	+++	+++
MyoD1 (b)	-	-	-	++	+++	+++	+++	+++	+++
Pax3 (c)	-	+	++	++	++	+	-	-	-
Pax7 (c)	-	-	+	++	++	++	++	++	++
MHox (c)	-	-	+	++	+	-	-	++	+++

(a) Desmin expression was characterized by immunocytochemistry (see text for references). (b) Expression of myogenic HLH regulatory factors was monitored by *in situ* hybridization. *In the case of *myogenin* and *MyoD* the corresponding protein was also detected by immunocytochemistry. Note that myogenin protein is not detected before 10.5 days of development while the corresponding RNA starts to be detected at 8.5 days. (c) Expression of paired homeobox genes was studied by *in situ* hybridization.

the level of the expression of their genes or they repress their activity by various mechanisms such as protein-protein interaction or phosphorylation resulting in the inability of myogenic HLH proteins to activate muscle-specific gene expression (Li *et al.*, 1992; Olson, 1992).

Lessons from the in vivo targeted inactivation of myogenic HLH regulators

In order to analyze the biological function of myogenic HLH factors, *in vivo* gene targeting experiments have been carried out by several laboratories.

Inactivation of the *MyoD* and *Myf-5* genes by homologous recombination does not result in dramatic alterations in the myogenic process (Braun *et al.*, 1992; Rudnicki *et al.*, 1992). The initial appearance of desmin and α-actin positive myotomes is delayed by several days in *Myf-5* deficient mice. These mice are also characterized by an abnormal rib development and die because they are unable to breathe. No abnormalities can be found in mice carrying mutated *MyoD* genes, which do reexpress *Myf-5* at stages where it is normally silent. Taken together, these data point to the existence of redundancy between these two different HLH factors (Braun *et al.*, 1992; Rudnicki *et al.*, 1992). This hypothesis was further tested by generating mice carrying null mutations in both the *Myf-5* and *MyoD* genes (Rudnicki *et al.*, 1993; Weintraub, 1993; Olson and Klein, 1994). Such mice are characterized by the almost complete absence of skeletal muscle. The lack of skeletal muscle appears to be due to the absence of determined myoblasts, characterized by the expression of desmin. Therefore it seems likely that *Myf-5* and *MyoD* are involved in the control of some critical steps in the myogenic determination process. However as myogenic precursor cells migrating from the dermomyotome to the limb do not express any of the known HLH myogenic factors, it is likely that *Myf-5* and *MyoD* are involved in some late decisional events in the determination process (Olson and Klein, 1994). These myogenic factors are not able to activate the expression of most genes encoding muscle differentiation markers in proliferating myoblast from the embryo. Therefore, much work will be needed to decipher the molecular mechanisms by which *Myf-5* and *MyoD* genes affect the determination process (Rudnicki *et al.*, 1993; Weintraub, 1993; Olson and Klein, 1994). The possibility that *Myf-5* and *MyoD* factors activate myoblast-specific genes through non-canonical target sequences by direct or indirect mechanisms must be considered. Other mechanisms might include the progressive inactivation of growth controlling factors such as the c-jun protein. Subsequently, the weak initial activation of a subset of target genes might become possible. These targets might include the *myogenin* gene whose promoter contains an HLH binding site which is required for its activation (Chang-Cheng *et al.*, 1992, 1993; Edmonson *et al.*, 1992; Yee and Rigby 1993). The myogenin gene product might subsequently trigger the transcriptional activation of muscle-specific genes.

Indeed, it was recently shown that *in vivo* inactivation of the *myogenin* gene leads to a failure of differentiation of the myoblastic cells into multinucleate myofibers in mice homozygous for the inactivating mutation, strongly suggesting that *myogenin* plays an essential role in the differentiation of myoblasts into myotubes (Hasty *et al.*, 1993). In these mutant mice only a few myofibers can be found (Hasty *et al.*, 1993).

All available data provide evidence that myogenic HLH factors play a key role in the late stages of myogenic cell determination and in their subsequent differentiation. A striking feature of the myogenic factor genes resides in their expression which appears to be restricted to the skeletal myogenic lineage. Although it might be argued that this specificity is due to the autoregulation of these genes by their own products, one must bear in mind that the initial activation of *Myf-5* gene transcription must require other regulators active at the very early stages of myogenic determination and which still remain to be identified. Evidence for myoblast-specific transcription independent of the myogenic HLH regulators has also been recently contributed by the observation that a myoblast-specific enhancer from the desmin gene is active in mononucleate myoblastic cells. This activity does not require the myogenic HLH factors but seems rather to be dependent on the binding of nuclear factors belonging to the Krox family of zinc fingers containing transcription factors (Li and Paulin, 1993 and unpublished observations). The wide distribution of these factors suggest that some unknown mechanisms must be involved in myoblast-specific transcription. On the other hand transcription of the desmin gene in myotubes appears to be dependent on a *bona fide* muscle-specific enhancer whose activity is controlled by myogenic HLH factors. It is also striking that *Myf-5* and *MyoD* deficient mice lack desmin expression. This leads us to the possibility that HLH independent myoblast-specific transcription is a characteristic of myotomal cells, whose phenotype is not stable in the absence of HLH regulators. This unstable phenotype might allow the conversion of these cells into adipocytes or fibroblast-like cells in *Myf-5* and *MyoD* deficient mice. On the other hand, limb myogenic precursor cells, which will migrate from the ventrolateral part of the dermomyotome to the limbs, retain their developmental capacities in the absence of myogenic factors. These factors will only be expressed after the cells have reached their final destination in the

limbs. Therefore, it seems that the stability of early myogenic phenotype might be dependent upon environmental factors and that one of the functions of the *Myf-5* and *MyoD* gene products might be to lock the determined state before pushing it toward a more advanced phenotype.

While little is known about the early stages of myogenic determination, many more data are available on the regulation of the late determination and differentiation programs. While myogenic HLH factors play a key role in these processes, there is increasing evidence that myogenesis requires their collaboration with more widely expressed factors.

Involvement of the SRF and MEF-2 MADS domain factors in the control of muscle-specific transcription

Dissecting the regulatory sequences of muscle-specific genes has provided evidence for a regulatory role for the CArG box binding factors, the MEF-2 and homeodomain containing proteins.

The CArG box ($CC(A/T)_6GG$) was first described as an evolutionary conserved motif, present in the upstream regions of a number of muscle-specific genes and in the serum response element (SRE) of the *c-fos* enhancer, which mediates serum induction of the transcription of this proto-oncogene (Treisman, 1985; Minty and Kedes, 1986; Phan-Dinh-Tuy *et al.*, 1988; Sartorelli *et al.*, 1990; Tuil *et al.*, 1990; Gilgenkrantz *et al.*, 1992). It was shown that one of the CArG box binding factors is identical to the ubiquitous serum responsive factor (SRF) which interacts with the SRE of the *c-fos* gene and of a number of genes activated in the G0-G1 transition (Norman *et al.*, 1988). Further experiments have clearly demonstrated that the SRF is involved in the control of both proliferation associated and muscle-specific genes in the three muscle types (skeletal, cardiac and smooth) (Norman *et al.*, 1988; Tuil *et al.*, 1990; Vandromme *et al.*, 1992). As cell proliferation prevents differentiation in the skeletal muscle lineage, the involvement of the same factor in these antagonistic phenomena is intriguing and it is clear that much work is needed to elucidate the molecular mechanisms at work (Santoro and Walsh, 1991; Trouche *et al.*, 1993). These studies are complicated by the growing list of proteins which interact with the SRF factor and by the occurrence of some factors which are not related to the SRF but still bind directly to the SRE.

MEF-2 factors were initially described as the products of a tissue-specific alternative splicing of at least three different ubiquitously expressed genes, generating isoforms carrying the MEF-2 activity, specifically in differentiated skeletal myotubes, cardiac and smooth muscle cells and brain (Gossett *et al.*, 1989; Pollock and Treisman, 1991; Yu *et al.*, 1992; Martin *et al.*, 1993, 1994). While the MEF-2 DNA-binding activity was shown by most investigators to be restricted to skeletal muscle, cardiac and smooth muscle and brain, some conflicting data indicate a more widespread distribution (Gossett *et al.*, 1989; Pollock and Treisman, 1991; Yu *et al.*, 1992; Martin *et al.*, 1994). A *Mef-2* related gene, *Mef-2C*, which is only expressed in terminally differentiated myotubes and in a subset of brain neuronal cells in the brain, was found to encode transcription factors highly related both structurally and functionally to MEF-2 factors (Martin *et al.*, 1993; McDermott *et al.*, 1993). Thus, it appears that members of the MEF-2 family can be generated by at least two tissue-specific mechanisms: alternative splicing or cell-specific transcription. Furthermore, the restricted occurrence of the MEF-2 DNA binding activity observed by most investigators might suggest that the expression of the proteins encoded by the ubiquitously transcribed *Mef-2* genes might also be controlled by translational or post-translational mechanisms. The MEF-2 factors, which bind to an A+T rich sequence (TAT/AAT/ATA/GAC) found in a number of muscle-specific control elements, are characterized by the presence of a MADS domain (Norman *et al.*, 1988; Gossett *et al.*, 1989; Pollock and Treisman, 1991; Yu *et al.*, 1992; McDermott *et al.*, 1993; Martin *et al.*, 1994). This domain was recently shown to be characteristic of a variety of transcription factors from diverse organisms, the prototype being the Serum Responsive Factor (SRF) (Norman *et al.*, 1988). The appellation MADS is derived from MCM1, *agamous*,

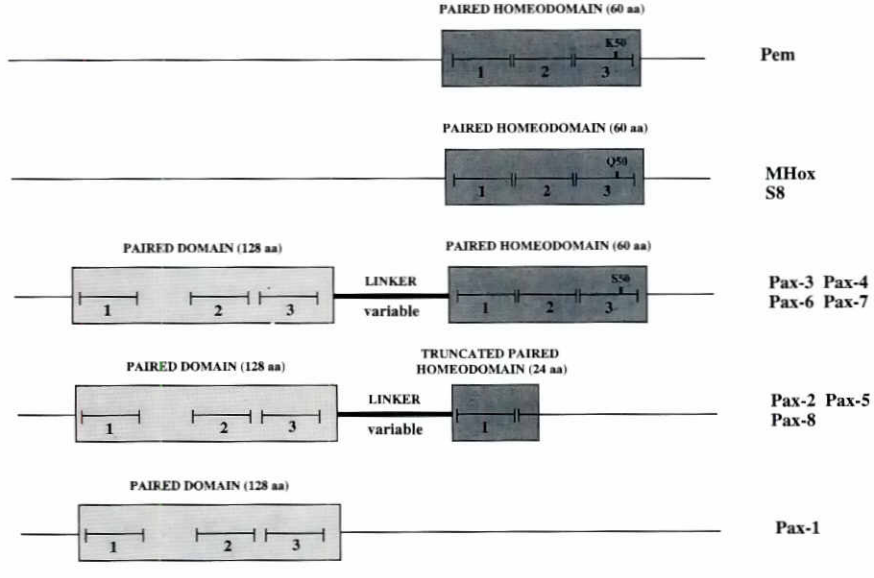

Fig. 2. Structural features of the different members of the "paired" family of transcription factors. *The two main classes of paired-like factors are depicted. The Pax class is characterized by the presence of the paired domain and can be further subdivided on the basis of the presence or the absence of a paired homeodomain. In addition to the paired domain Pax-3, 4, 6, and 7 contain a complete paired homeodomain characterized by the presence of a serine residue at the position 50, while Pax-2, 5 and 8 contain only a truncated paired homeodomain of 24 aminoacids. The Pax-1 gene product only contains a paired domain. The other class of paired-like proteins is lacking the paired domain and only contains a paired homeodomain, characterized by the presence of either a glutamine or a lysine residue at its position 50. Two characteristic members of this class, which are expressed in myogenic cells, are depicted. The MHox protein and the related S8 gene product contain a paired homeodomain with a glutamine residue at position 50 while, in the Pem protein, the paired homeodomain contains a lysine at this position. So far paired homeodomains carrying a serine residue at the position 50 have always been found to be associated with paired domains in the same protein.*

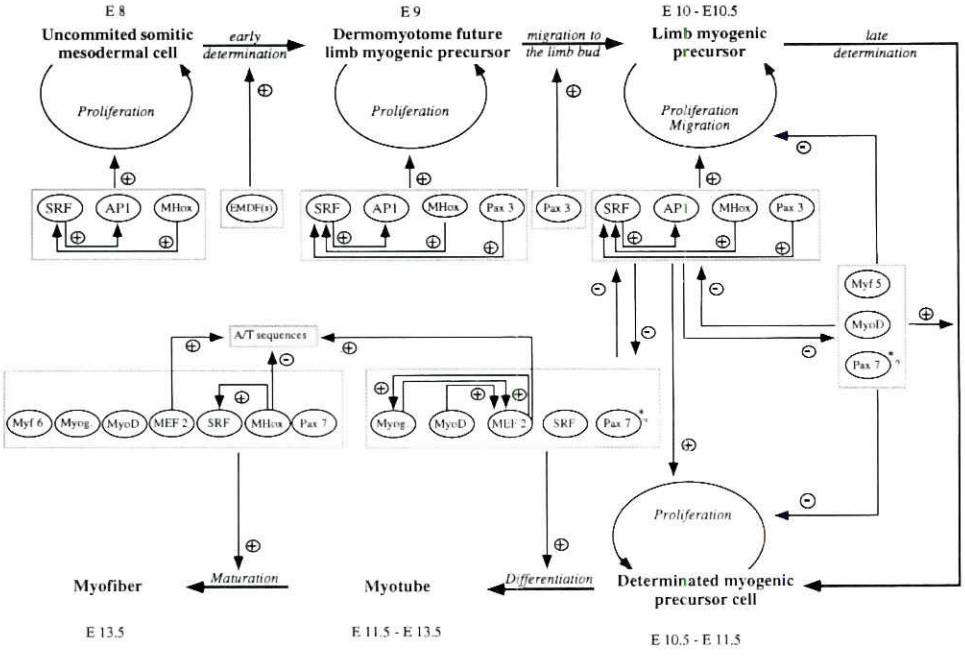

Fig. 3. A model for the functional relationships between myogenic regulatory factors in the developing limb of the mouse embryo. *A detailed description of the model can be found in the text. E9: day 9 of embryonic development; Myog: myogenin; ?*Pax-7 expression has not been documented in the limb at this stage. However due to the similarities between Pax-7 and myogenic factor gene expression in trunk muscle, we assume that Pax-7 is expressed in the limb at this stage*

deficiens and SRF, which are the first discovered transcription factors containing this DNA-binding domain (Norman *et al.*, 1988). MCM1 is a yeast factor, which is involved in yeast sexual differentiation, while *agamous* and *deficiens* are plant homeotic genes (Norman *et al.*, 1988).

MEF-2 factors and SRF are the products of different genes and interact with different target sequences (Norman *et al.*, 1988; Santoro and Walsh, 1991; Vandromme *et al.*, 1992; Yu *et al.*, 1992; McDermott *et al.*, 1993; Martin *et al.*, 1994). Thus, at least two different types of MADS domain factors are involved in the control of muscle-specific transcription. MEF-2 binding sites are often found in the vicinity of target sequences for myogenic HLH factors and both types of factors can cooperate in the activation of skeletal muscle-specific transcription, raising the possibility of interactions between both types of factors (Edmonson *et al.*, 1992; Olson, 1992; Li and Paulin, 1993). Nevertheless, MEF-2 proteins are able to transactivate natural and artificial promoter constructs carrying MEF-2 binding sites in non myogenic cells and skeletal myoblasts in the absence of HLH binding sites (Gossett *et al.*, 1989; Yu *et al.*, 1992; Martin *et al.*, 1994). However, their ability to convert C3H/10T1/2 fibroblastic cells into myogenic cells has not yet been reported. MEF-2 is induced by forced expression of MyoD and myogenin, suggesting that it lies downstream of the myogenic HLH proteins in a dependent regulatory pathway (Cserjesi and Olson, 1991; Olson, 1992). Interestingly, the *myogenin* promoter contains both a MEF-2 site and a target sequence for myogenic HLH proteins that collaborate to impart muscle-specificity and positive autoregulation *in vitro* in cultured cells and spatio-temporal expression *in vivo* in transgenic mice (Chang-Cheng *et al.*, 1992, 1993; Edmonson *et al.*, 1992; Yee and Rigby, 1993).

During mouse embryonic development, *Mef-2C* transcripts are the first to appear, followed by those originating from the other *Mef-2* genes, which can be detected half a day later (Edmonson *et al.*, 1994). *Mef*-2C transcripts are first detected at day 7.5 in cardiac muscle precursors and appear shortly before day 9 in somites where their expression is then restricted to the myotomes. Transcripts from the other *Mef-2* genes are also detected in non-muscle cell types, such as migrating neural crest cells, consistent with their wide expression in adult tissues.

The relatively late expression of *Mef-2* transcripts *in vivo* in skeletal muscle precursors argues against the hypothesis of the involvement of their protein products in the initial activation of the *myogenin* gene. On the other hand, MEF-2 factors might play a role in the subsequent accumulation of *myogenin* transcripts. Therefore, it seems likely that the HLH binding site from the *myogenin* promoter plays a key role in the initial activation of this gene.

The initial analysis of MEF-2 factors *in vitro*, in cultured cells, suggested that they could be acting at a similar level to the myogenic HLH factors in the hierarchy of myogenic factors. The *in vivo* expression data argue against this hypothesis and rather suggest that MEF-2 factors are initially induced by a low amount of myogenic factors. Subsequently they enhance the transcription of the myogenic factor genes thus leading to a positive auto-regulatory loop, which provides the myogenic cells with the required amount of both myogenic HLH and MEF-2 factors which cooperate to activate the transcription of downstream genes.

The isolation and characterization of the MEF-2 factors provides a successful example of the identification of cell-specific regulators through classical analysis of the transcription of genes encoding differentiation markers. The use of the same strategy enabled the isolation of a new paired homeodomain protein, which binds a critical site in a muscle-specific enhancer.

Paired homeodomain proteins and muscle-specific transcription: possible involvement of both direct and indirect mechanisms

Biphasic expression of the paired-like homeobox gene MHox in the skeletal myogenic lineage

An A+T rich region essential for the activity of the myotube-specific enhancer of the muscle-specific creatine kinase *(MCK)* gene was recently described (Cserjesi *et al.*, 1992). This sequence

is not able to activate muscle-specific transcription alone. However, mutagenesis of this sequence has shown that its integrity is an essential requirement for the activity of the enhancer. This appears to result from the functional cooperation of myogenic HLH factors binding to two adjacent target sequences with factor(s) interacting with the A+T rich element (Cserjesi *et al.*, 1992).This element, which interacts poorly with pure MEF-2, was found to be a binding site for at least two other factors, which led to the hypothesis of an interaction between these two DNA binding proteins and the MEF-2 factors resulting in the optimal binding to this element (Cserjesi *et al.*, 1992). Screening of a myotube specific cDNA expression library yielded a cDNA clone encoding a new homeodomain protein referred to as MHox, corresponding to the major DNA-protein complex observed upon incubation with nuclear extracts (Cserjesi *et al.*, 1992).

The homeodomain is a 60 aminoacids helix-turn-helix DNA-binding motif initially discovered in the products of *Drosophila* developmental control genes (Kessel and Gruss, 1990). More than 20 different types of homeodomains have been found in protein products of genes involved in the control of a variety of biological processes. These genes include developmental control genes acting at various levels such as positional information or stage specific differentiation in both insects and vertebrate organisms (Kessel and Gruss, 1990).

In the case of the MHox gene product, available data show that both endogenous (from myotubes) and cloned MHox factor specifically interact with the A+T rich element (Cserjesi *et al.*, 1992). As DNA motifs similar to the MCK MHox binding site have been found in the control regions of other muscle-specific genes, it was suggested that this factor could take part in the coordinate regulation of gene expression during myogenesis (Cserjesi *et al.*, 1992). As the MHox target sequence is also a weak binding site for the MEF-2 factor, one might ask whether the MHox homeodomain protein and the MEF-2 MADS domain factors do interact with each other. This hypothesis is supported by the independent cloning of the *MHox* gene which was based on the ability of its protein product to functionally interact with the yeast MADS domain specific factor MCM1 (Grueneberg *et al.*, 1992). In this work, MHox, referred to as Phox, was further shown to functionally interact with yeast MCM1 and mammalian SRF MADS domains and enhance their binding to their target sequences (Grueneberg *et al.*, 1992). It was shown that only the MHox homeodomain is required for this interaction with the MADS domain (Grueneberg *et al.*, 1992), which was recently shown to be direct and to require aminoacid residues within the second a-helix of the paired homeodomain (Grueneberg *et al.*, 1993).

Further experiments have shown that MHox is able to transactivate promoters containing the *c-fos* SRE, suggesting that the MHox protein is able to enhance the expression of the *c-fos* gene (Grueneberg *et al.*, 1992). The c-fos gene product has been shown to inhibit skeletal muscle differentiation. That's why additional mechanisms, such as transcriptional repression by myogenic HLH factors, must ensure that *c-fos* gene expression is down-regulated prior to differentiation, while muscle-specific promoters containing CArG-SRE boxes become fully active at this stage (Santoro and Walsh, 1991; Trouche *et al.*, 1993).

MHox expression was first detected at day 9 in many mesenchymal cells in the mouse embryo and found to be mostly restricted to skeletal muscle and also to some smooth muscles in the adult mouse (Table 1) (Cserjesi *et al.*, 1992; Kern *et al.*, 1992). Adult skeletal muscle expresses high levels of *MHox* mRNA and protein (Cserjesi *et al.*, 1992; Kern *et al.*, 1992). Some *MHox* transcripts can be detected in adult heart (Cserjesi *et al.*, 1992). However, they seem to originate from pericardial tissue rather than from cardiac muscle (Kern *et al.*, 1992). Thus, *MHox* expression is characteristic of some muscle tissues in late gestation embryos and adult mice.

On the other hand, detailed analysis of its early expression in both murine and chicken embryos, has shown that it is only transiently expressed in structures containing skeletal myogenic precursors (Cserjesi *et al.*, 1992; Kuratani *et al.*, 1994). Indeed, its expression can be detected in somites at day 9 in the mouse embryo and at a comparable developmental stage in chick (Cserjesi *et al.*, 1992; Kuratani *et al.*, 1994). In the somites, *MHox* expression is restricted to the dermomyotome. The dermomyotome contains precursors of trunk skeletal muscle, limb skeletal muscle and dermis (Christ *et al.*, 1986). At a slightly later stage, when the dermomyotome has given rise to the myotome and the dermomyotome, *MHox* expression is found to be restricted to the dermatome and later on to the embryonic dermis (Kuratani *et al.*, 1994). No expression of *MHox* will be detected in trunk skeletal myogenic cells until late developmental stages corresponding to myofiber maturation (Cserjesi *et al.*, 1992; Kuratani *et al.*, 1994).

In the newly formed limb buds from both murine and chicken embryos, all mesenchymal cells express *MHox*. Therefore, it seems likely that the mononucleate limb myogenic precursor cells that have migrated from the dermomyotome to the forming limb buds, express *MHox* at this stage. However, it is not yet known whether *MHox* is expressed in these precursors when they migrate from the dermomyotome to the limb buds. At slightly later stages, *MHox* expression switched off in myogenic limb precursor cells and in the resulting myotubes. At late gestation stages, *MHox* is reexpressed in maturating limb muscle fibers, similar to the situation found in trunk muscle.

In other mesenchymal structures, a very strong *MHox* expression can be detected in neural crest-derived mesenchymal cells from the first branchial arch (Cserjesi *et al.*, 1992; Kuratani *et al.*, 1994). This might suggest an involvement of the MHox gene product in the development of craniofacial structures (Couly *et al.*, 1993; Le Douarin *et al.*, 1993). While *MHox* expression is never detected in the sclerotome and prevertebrae, it is transiently expressed in precartilaginous structures from the limbs and ribs, where its expression is switched off upon chondrocyte maturation (Cserjesi *et al.*, 1992; Kuratani *et al.*, 1994).

This complex expression pattern suggests that MHox might differentially control gene expression at various stages of development. In undifferentiated mesenchymal cells and migrating limb myogenic precursors, MHox might be involved in the control of the expression of a set of genes characteristic of the proliferative and migratory states of these cells. This control might involve both binding of the MHox protein to specific target DNA sequences and functional interaction of the MHox gene product with the SRF MADS domain containing protein, such as the SRF in undifferentiated mesenchymal cells and limb myogenic precursors.

Possible target genes for MHox might then include multiple genes carrying functional SRE-like sequences in their control regions. Such genes might include the *c-fos* protooncogene and the gene encoding the zinc fingers containing transcription factor Krox-24-*Zif*-268-Egr-1, which does carry multiple functional SRE-like elements in its promoter and exhibits an expression pattern similar to *MHox*, being expressed in embryonic mesenchymal cells

and in all three types of muscles (McMahon *et al.*, 1990). Interestingly, a myoblast specific enhancer involved in the control of the myogenic determination and differentiation marker desmin contains several functional Krox-24 binding sites which are important for their activity in proliferating myoblasts (Li and Paulin, 1993). Krox-24 was also shown to upregulate the expression of the rat cardiac a-myosin heavy chain gene in cardiac muscle cells (Gupta *et al.*, 1991).

As cell proliferation is antagonistic to the final steps of the myogenic determination-differentiation process, *MHox* expression might have to be shut off in order to allow these late decisional events. At much later stages of development, both growth stimulation and expression or activity of growth-promoting nuclear regulators such as c-fos and c-jun have been turned off by various mechanisms. Therefore, *MHox* expression would not be able to enhance these already shut off processes. *MHox* reexpression could reflect the recruitment of its product in completely different processes such as myofiber maturation through the same molecular mechanisms. This reexpression is correlated with the increased expression of the *MCK* gene (Cserjesi *et al.*, 1992). This might be due to an increased activity of the *MCK* enhancer at least in part through the A+T rich MHox-binding site (Cserjesi *et al.*, 1992). The postulated MHox-mediated increase in the activity of the *MCK* enhancer could also be mediated by indirect mechanisms. These could include interactions with the SRF and MEF-2 MADS domain transcription factors. Such an interaction could lead to a further enhancement of the enhancer activity through the CArG and MEF-2 motifs from the MCK enhancer. The occurrence of MHox, CArG and MEF-2 motifs in many muscle specific genes favors the hypothesis that MHox could modulate the transcription of these genes in maturating muscle fibers (Cserjesi *et al.*, 1992). We would like to point out that the relatively late onset of *MHox* reexpression suggests an involvement in subtle molecular events related to terminal maturation of the myofiber but not in its formation. Therefore one would expect *MHox* to modulate already set up processes such as *MCK* enhancer activity. While the above hypothesis is based n the assumption that the MHox protein is involved in the positive regulation of the activity of muscle-specific regulatory elements such as the *MCK* enhancer, the hypothesis of a negative regulatory function for the MHox factor must also be considered. Indeed, the A+T rich MHox-binding sequence found in the *MCK* enhancer and in the regulatory regions of other muscle-specific genes is able to interact both with MEF-2 and MHox proteins (Cserjesi *et al.*, 1992, 1994). It was also shown that MEF-2 factors are able to stimulate the activity of reporter constructs containing several copies of the A+T rich sequence in non myogenic cells (Cserjesi *et al.*, 1994). As the ability of the MHox protein to enhance transcription through this sequence could not be demonstrated, this would suggest that MHox could compete with MEF-2 for a common A+T rich target sequence and thus prevent transcriptional activation by the MEF-2 factor through a subset of its target sequences. MHox expression in maturating muscle fibers would then be part of a mechanism which would prevent MEF-2 induced overexpression of a set of muscle-specific genes carrying the A+T rich sequence.

The functional cooperation between the MHox and myogenic HLH binding sites of the *MCK* enhancer led to the suggestion that the MHox product collaborates with the myogenic HLH factors to further enhance muscle-specific gene transcription (Cserjesi *et al.*, 1992). This hypothesis was supported by the observation that mesodermal cell lines which express *MHox* exhibit the best response to the introduction of exogenous myogenic HLH proteins leading to the switching on of the muscle differentiation program. Indeed, other cell types exhibit little or no myogenic conversion in response to the introduction of these factors, thus supporting the hypothesis of a cooperation between MHox and HLH factors for the optimal induction of a fully differentiated myogenic phenotype (Lassar *et al.*, 1987; Schäfer *et al.*, 1990; Buckingham, 1992; Cserjesi *et al.*, 1992; Olson, 1992). On the other hand, binding of the MEF-2 factors to the MHox target sequence could also be a likely explanation for the observed cooperation between MHox and HLH binding sites as the functional cooperation between MEF-2 and myogenic factors is well established. We would therefore like to suggest that the MHox protein is part of a negative regulatory mechanism which limits the activation of a subset of muscle specific genes in both undifferentiated myogenic precursors and in maturating muscle fibers through binding to the A+T rich sequence. On the other hand, the functional interaction of MHox with the SRF might involve both in the repression of the initial steps of muscle differentiation by turning on immediate early genes such as *c-fos* and in the enhancement of muscle-specific transcription through the CArG boxes in maturating myofibers.

Paired homeodomain proteins and MADS domain factors

Analysis of the predicted amino acid sequence of the MHox homeodomain shows a very high percentage of homology to the homeodomain of the product of the *S8* gene which is also expressed predominantly in embryonic mesenchyme, but not in muscle (Opstelten *et al.*, 1991; Cserjesi *et al.*, 1992; Kern *et al.*, 1992). Figure 2 shows that MHox and S8 homeodomains bear significant homology to the homeodomain of the Drosophila regulatory protein paired (Fig. 2). However, unlike *Drosophila* paired proteins, containing both a paired domain and a paired homeodomain, S8 and MHox proteins do not contain any paired domain and are characterized by the presence of a glutamine residue at position 50 of their homeodomains. Indeed they recognize target sequences related to the targets of Antp-like homeoproteins, such as the products of the vertebrate *Hox* genes (Fig. 2) (Jaynes and O'Farrell, 1988; Treisman *et al.*, 1989; Kessel and Gruss, 1990; Goulding *et al.*, 1991; Jostes *et al.*, 1991; Opstelten *et al.*, 1991; Chalepakis *et al.*, 1992; Cserjesi *et al.*, 1992). The MHox target sequence contains the motif TATTAAT which is also found in the binding sites for the neural homeoproteins HoxC6 and Phox-2 in the N-CAM promoter (Jones *et al.*, 1993; Valarché *et al.*, 1993). Like MHox, the Phox-2 gene product contains a paired-like homeodomain with a glutamine residue at position 50 (Valarché *et al.*, 1993). In this respect, they both differ from *Drosophila* paired and gooseberry gene products and from their vertebrate counterparts, the Pax-3, 4, 6 and 7 proteins which are characterized by the presence of a serine residue at position 50 of their homeodomains. Indeed, the nature of this 50th residue, located in the DNA recognition helix of the homeodomain has been shown to determine the DNA-binding specificity of homeodomain proteins (Treisman *et al.*, 1989). Antp-like homeodomains do not interact with the SRF, while various paired-like homeodomains carrying either a serine, a lysine or a glutamine at position 50, do so, suggesting that this interaction is a common feature of paired homeodomains irrespective of their target DNA sequences (Grueneberg *et al.*, 1992). Interestingly, the expression of the *Pem* gene encoding another paired homeodomain protein, characterized by the presence of a lysine residue at position 50 of its homeodomain (Fig. 2), was found to be upregulated upon *in vitro*

myogenic conversion of C3H/10T1/2 cells induced by 5-azacytidine (Wasaki *et al.*, 1991). Furthermore, high *Pem* expression was also observed in stable myoblast cell lines derived from converted cells and in differentiated myotubes obtained from these lines (Wasaki *et al.*, 1991).

More interestingly, two members of the *Pax* multigenic family, *Pax-3* and *7*, have been shown to be expressed both in the nervous system and the developing skeletal muscle (Goulding *et al.*, 1991; Jostes *et al.*, 1991). Pax-3 and 7 proteins, which are the products of highly related genes, contain a paired homeodomain. Therefore they could be part of a network of paired homeodomain factors active in myogenic cells.

The Pax family of developmental control transcription factors

Recent investigations have led to the conclusion that *Pax* genes encode transcription factors which interact specifically with target DNA sequences and are able to modulate the transcription of genes carrying such motifs (Goulding *et al.*, 1991; Chalepakis *et al.*, 1991, 1992). Available data with *Pax-5* and *8* further suggest that *Pax* genes are involved in the control of the expression of genes encoding differentiation markers characteristic of the structures that do express *Pax* genes (Chalepakis *et al.*, 1992). Furthermore, there is clear evidence that Pax gene products are involved in the control of specific developmental processes (Chalepakis *et al.*, 1991, 1992; Epstein *et al.*, 1991; Tassabehji *et al.*, 1993). Indeed, analysis of spontaneous mutations in both murine and human *Pax-3* and *6* genes has clearly demonstrated that these genes are involved in the development of the nervous system, where they are expressed. Mutations within the *Pax-1* gene affect the formation of the developing vertebral column, where this gene is expressed (Chalepakis *et al.*, 1991, 1992; Epstein *et al.*, 1991; Tassabehji *et al.*, 1993).

Analysis of the structure of the Pax proteins enables their classification into two main groups according to the presence or the lack of a complete paired type homeodomain (Fig. 2) (Chalepakis *et al.*, 1992). Pax-3, Pax-4, Pax-6 and Pax-7 gene products are characterized by the presence of both a 128 amino acids paired domain and a paired homeodomain in the same protein. This results in the presence of two potentially independent sequence-specific DNA binding domains in the same transcription factor. In this respect, they differ from the other members of the *Pax* multigenic family (Pax-1, Pax-2, Pax-5 and Pax-8) lacking most if not all of the homeodomain sequences but containing a paired domain, which constitutes an independent DNA binding domain (Fig. 2) (Goulding *et al.*, 1991; Chalepakis *et al.*, 1991, 1992). As the homeodomain of the *Drosophila* Paired protein, which is closely related to that of the vertebrate Pax-3, 4, 6 and 7, does functionally interact with the MADS domains of the MCM1 and SRF factors, it is very likely that Pax-3, 4, 6 and 7 proteins do also have this property (Chalepakis *et al.*, 1991, 1992; Goulding *et al.*, 1991; Grueneberg *et al.*, 1992). This hypothesis is supported by recent data, showing a transactivation of the *c-fos* promoter by the Pax-6 gene product in the absence of serum (Chalepakis *et al.*, 1992). This transactivation seems to be mediated by several distinct target sequences in the *c-fos* promoter, one of which has been shown to bind the Pax-6 protein (Chalepakis *et al.*, 1992). Due to the presence of a paired homeodomain related to that of the *Drosophila* Paired protein in the Pax-6 protein, it might be suggested that this protein does also act through the SRE by interacting with the SRF. However, this hypothesis remains to be tested experimentally (Chalepakis *et al.*, 1992; Grueneberg *et al.*, 1992).

Such experiments could not be performed with the Pax-4 and 7 gene product as their complete coding sequence is not yet available. The ability of the paired homeodomain Pax-3 protein to influence the activity of the c-fos promoter in any direction was not reported. As its paired homeodomain exhibits a higher homology to the paired homeodomain of the *Drosophila* Paired gene product than the Pax-6 homeodomain, the possibility remains open that the Pax-3 protein interacts with the SRF to influence positively or negatively the activity of the *c-fos* promoter (Goulding *et al.*, 1991; Chalepakis *et al.*, 1992; Grueneberg *et al.*, 1992). It is noteworthy that a lack of effect of the Pax-3 protein on the activity of this promoter in co-transfection experiments would not necessarily imply that Pax-3 does not play any role in the control of *c-fos* transcription. Indeed, several reports have shown that several homeodomain proteins are unable to modulate transcription of promoters containing their target sequences, but can still prevent their activation by other homeodomain proteins by a competition mechanism (Jaynes and O'Farrell, 1988). Therefore the Pax-3 gene product and possibly the related Pax-7 protein might either prevent activation of the *c-fos* gene by other paired homeodomain factors or rather enhance the activity of the *c-fos* regulatory region. While experimental evidence for positive or negative modulation of *c-fos* gene expression by the Pax-3 protein has not been reported, the oncogenic potential of a deregulated *Pax-3* gene does argue in favor of a positive modulation of *c-fos* transcription (Maulbecker and Gruss, 1993).

The Pax-3 gene and early myogenesis

Most interest has been devoted to the expression of *Pax-3* and *7* genes in the developing nervous system. Indeed, both genes are expressed in the dorsal part of the embryonic neural tube and in specific areas of the brain (Goulding *et al.*, 1991; Jostes *et al.*, 1991). Additionally *Pax-3* is also expressed in the neural crest. These *Pax* genes are also expressed in mesodermal cells during mouse embryogenesis. *Pax-3* expression was found to occur in the somite and later on in its dermomyotomal compartment between days 8.5 and 11 of mouse embryonic development (Table 1) (Goulding *et al.*, 1991; Dietrich *et al.*, 1993), which is quite reminiscent of the transient expression of *Myf-5* in this structure between embryonic days 8 and 12 (Table 1) (Buckingham, 1992). However *Pax-3* expression domain is wider than *Myf-5* domain as it is expressed not only in the future myotome but also in dermomyotomal ventrolateral cells, which give rise to the limb muscle precursors (Bober *et al.*, 1994). Indeed, *Pax-3* expression can be detected in cells that migrate from this structure to the forelimb limb buds at day 9.5 (Bober *et al.*,1994). Subsequently *Pax-3* expressing cells are found in the limb bud at day 10 before the appearance of myogenic factors in this structure (Goulding *et al.*, 1991; Bober *et al.*, 1994). Analysis of *Pax-3* gene expression in chicken embryo yielded similar results (William and Ordahl, 1994) Therefore, *Pax-3* is a marker for the limb myogenic precursor cells. Detailed analysis of *Splotch* mice and of patients suffering from the type III form of human Waardenburg syndrome, which are characterized by the presence of a mutated or deleted *Pax-3* gene, has revealed alterations in the formation of skeletal muscles of the limbs, which do not form in homozygous *Splotch* mice (Epstein *et al.*, 1991; Franz *et al.*, 1993; Tassabehji *et al.*, 1993; Bober *et al.*, 1994). However, as little if any abnormalities can be observed in trunk muscle, it is still premature to conclude that *Pax-3* is involved in the control of muscle-specific transcription.

Human *PAX-3* transcripts are detected in several rhabdomyosarcoma cell lines, which are believed to originate from satellite myoblast cells (Galili *et al.*, 1993). The presence of *PAX-3* transcripts in these undifferentiated muscle tumors does support the hypothesis of a functional role for the *Pax-3* gene in undifferentiated committed myogenic cells (Galili *et al.*, 1993). In alveolar rhabdomyosarcoma carrying a t(2;13)(q35;q14) translocation, the human *PAX-3* gene was found to be the rearranged chromosome 2 locus.The t(2;13)(q35;q14) translocation results in the fusion of chromosome 2 sequences encoding for the N-terminal portion of the PAX-3 product, including the paired domain and the paired homeodomain to another locus from the chromosome 13 (Galili *et al.*, 1993). This chromosome 13 locus encodes for a putative transcription factor, referred to as FKHR, characterized by the presence of a fork head domain. In alveolar rhabdomyosarcoma, the translocation results in the formation of a fusion protein consisting of the intact PAX-3 DNA-binding domain and the distal half of the fork head domain and C-terminal part of the FKHR gene product. These observations suggest that a rearranged *PAX-3* gene could be involved in the genesis of these tumors.

The hypothesis of an oncogenic potential of the *PAX-3* gene product is supported by the recent observation that overexpression of several murine *Pax* genes, including *Pax-3*, in normal fibroblastic cell lines is able to induce oncogenic transformation of cell cultures and tumor formation in mice (Maulbecker and Gruss, 1993). These observations favour the hypothesis of an involvement of the Pax-3 gene product in the control of the proliferation of myogenic precursor cells. This hypothesis is further supported by the observation that, in the embryonic neural tube, *Pax-3* is expressed by neuroblasts of the ventricular zone, which undergo active proliferation and migration. This observation does also suggest that the Pax-3 protein could be involved in the control of cell migration (Goulding *et al.*, 1991). Such a hypothesis is supported by the lack of *Pax-3* positive migrating dermomyotomal cells in *Splotch* mice (Bober *et al.*, 1994). This possible involvement of Pax-3 in the control of migratory processes is also supported by the analysis of *Splotch* mice which lack some structures originating from neural crest cell migration (Epstein *et al.*, 1991; Franz *et al.*, 1993; Tassabehji *et al.*, 1993) and by the observation that the migration of neural crest cells from *Splotch* neural tube explants is delayed (Moase and Trasler, 1990). Analysis of the rat *Small Eye* mutation, which affects another *Pax* gene, *Pax-6*, also shows defects in neural crest cell migration (Matsuo *et al.*, 1993). Therefore, it seems that at least two Pax genes could control cell migrations. Thus, the lack of limb muscles in the limbs of *Splotch* mice could be due either to a lack of migration of somitic myogenic precursors to the limbs or to a lack of proliferation of precursors in the limbs after completion of migration; it may even be due a to a combination of both mechanisms.

The Pax-7 gene and myogenesis

Pax-7 is expressed in both the embryonic nervous and muscular systems (Jostes *et al.*, 1991). In the muscular system, *Pax-7* expression appears at day 9 in the dermomyotome and is then restricted to the myotome, whereas the dermatome does not express this gene (Table 1) (Jostes *et al.*, 1991). Later during development, *Pax-7* transcripts are found in skeletal muscle cells, which derive from the myotome, but not in cardiac and smooth muscle cells, whose origin is different (Table 1) (Jostes *et al.*, 1991). Strikingly, *Pax-7* transcripts are expressed by both the determined myogenic precursors and the differentiated skeletal muscle cells (Jostes *et al.*, 1991). Thus, *Pax-7* expression is correlated with the muscle cell lineage. In this respect, it is very similar to the expression profile of the regulatory gene encoding *myogenin* and the muscle-specific intermediate filament desmin gene during skeletal muscle development (Table 1) (Buckingham, 1992; Chang-Cheng *et al.*, 1992, 1993; Olson, 1992; Li *et al.*, 1993; Yee and Rigby, 1993). This observation might suggest that all three gene regulatory regions share common control elements which could interact with regulators present at this stage or earlier in the formation of skeletal muscle precursors. However, both desmin and *Pax-7* expression profiles are more complex than *myogenin* transcript distribution. Desmin is also expressed in cardiac and smooth muscle cells *in vitro* and *in vivo* (Jostes *et al.*, 1991; Buckingham, 1992; Li *et al.*, 1993), while *Pax-7* transcripts are also detected in the nervous system. In the case of desmin, it was shown that 1 Kb of upstream sequences, containing a skeletal muscle specific enhancer, are necessary and sufficient for the generation of a skeletal muscle lineage specific expression pattern in transgenic mice similar to the profiles of the endogenous desmin, *myogenin* and *Pax-7* genes expression in developing skeletal muscles (Jostes *et al.*, 1991; Buckingham, 1992; Li *et al.*, 1993). Interestingly, transgene expression starts to be detectable at day 9; this raises the question as to whether *Pax-7*, whose expression starts at the same time, is involved in the cell-specific activity of the desmin transgene (Li *et al.*, 1993). No expression was detected in smooth and cardiac muscle using the 1 Kb desmin construct, suggesting the existence of other control elements localized in another part of the gene (Li *et al.*, 1993). Analysis of the DNA sequences involved in both skeletal muscle and nervous specific expression of the *Pax-7* gene will reveal whether these different expression profiles are controlled by different regulatory elements. Such an observation was recently made for the murine homeobox *En-2*, whose expression patterns in the midbrain-hindbrain junction and in mandibular myoblasts are controlled by two different enhancer elements (Logan *et al.*, 1993). As the expression of *myogenin-Lac-Z* transgenes, carrying either 1565 or 1072 bp, can first be detected between days 8.5 and 9, prior to the expression of the *Pax-7* gene, it is unlikely that Pax-7 plays a role in the initial activation of the myogenin gene (Chang-Cheng *et al.*, 1992, 1993; Yee and Rigby, 1993). However, the possibility remains open that the Pax-7 gene product could play a role in the subsequent increase in the transcription of the *myogenin* gene.

Although *Pax-7* gene expression pattern could suggest an involvement of its product in the molecular control of muscle-specific transcription, we must consider the possibility that the Pax-7 protein is rather implicated in the regulation of proliferative and migratory processes like the related Pax-3 gene product. This would involve the positive or negative modulation of the transcription of genes whose protein products play an active role in these phenomena. Such putative targets might include genes encoding for cell adhesion molecules such as the neural cell adhesion molecule (N-CAM) gene. N-CAM expression is detected in somites and in their derivatives, including myogenic cells, and in neural tissue (Thiery *et al.*, 1982; Moore *et al.*,1987; Moase and Trasler, 1991). Some indirect data showing an increase in the level of N-CAM protein expression in *Splotch* mice and more recent co-transfection experiments showing a transactivation of the *N-CAM* promoter by the Pax-6 protein suggest that *N-CAM* gene expression could be negatively controlled by the Pax-3 gene product (Epstein *et al.*, 1991; Moase and Trasler, 1991; Chalepakis *et al.*, 1992). Due to the homology between Pax-3 and Pax-7 gene

products, this would imply that the *N-CAM* gene is a potential target for the Pax-3 and 7 proteins.

Recent expression data obtained with a chicken *Pax-7* probe have demonstrated that *Pax-7* transcripts are downregulated in the dermomyotome cells which migrate toward the limb buds (Goulding *et al.*, 1994). Therefore *Pax-7* gene expression and myogenic precursor cell migration are mutually exclusive. Later during development *Pax-7* is expressed in differentiating limb muscle cells like the myogenic factors genes. We would like to suggest that one of the function of the Pax-7 gene product might be to control negatively cell migration and proliferation which are antagonistic to muscle cell differentiation. This would imply that the Pax-7 gene product and the myogenic HLH factors somehow cooperate to trigger limb muscle cells differentiation. It was shown that N-CAM expression is downregulated in migrating cells and reinduced as the cells stop migrating (Thiery *et al.*, 1982). Therefore, the Pax-7 gene product might be involved in the increase in *N-CAM* gene expression, which is characteristic of non migrating differentiating myogenic cells (Moore *et al.*, 1987). It is noteworthy that such a hypothesis would imply that the Pax-3 and Pax-7 gene product have antagonistic functions. Further experiments such as the analysis of the transcriptional regulatory properties and the oncogenic potential of the Pax-7 gene product will be required to test the hypothesis.

Analysis of the phenotypes induced by *Pax* gene mutations in mice and human has provided evidence for the loss of specific structures, thus supporting the hypothesis that the Pax gene products positively control cell proliferation. The obtention of mice carrying inactivated *Pax-7* alleles will provide a decisive tool to analyze the function of the Pax-7 gene product. Our model does predict abnormal increase of some structures expressing the *Pax-7* gene, which might be apparent in these mutant mice.

Conclusion and perspectives

A major impetus in the understanding of skeletal muscle-specific transcription was brought about by the discovery of the MyoD family of myogenic HLH transcription factors. However, although many aspects of muscle gene expression can be explained by the involvement of this class of regulators and the control of both their expression and activity, it has become clear that they need to cooperate with other types of regulators to achieve a proper control of the myogenic differentiation program. Unlike the myogenic HLH factors, which are only expressed in skeletal muscle, the other regulatory factors are expressed in several cell types.

MADS domain factors such as the ubiquitous SRF and the muscle and brain-specific MEF-2 proteins appear to play an essential role in the myogenic process. At the early stages of development, the SRF appears to be involved in the control of the proliferation of undifferentiated myogenic through transcriptional activation of immediate early genes such as *c-fos*. At later stages, the same factor could stimulate muscle-specific gene expression mediated by CArG boxes. Thus, the same factor could be involved in the positive control of two mutually exclusive phenomena, proliferation and differentiation. On the other hand, the MEF-2 factors are involved in the positive control of muscle-specific gene transcription and cooperate with myogenic HLH factors to stimulate the muscle differentiation program.

Paired homeodomain proteins such as MHox, Pax-3 and 7 genes products could play a role in more restricted processes such as limb myogenic precursor cell proliferation and migration, which are very likely to be controlled by the Pax-3 gene product. While no functional data are available for *Pax-7*, careful analysis of its expression pattern suggests that it could negatively control the migration and proliferation of the myogenic precursor cells and thus cooperate with the myogenic HLH and the MEF-2 factors to trigger muscle differentiation. While the activities of Pax gene products are generally thought to be mediated by their specific binding to target DNA sequences, the Pax-3 and 7 proteins could also interact with the SRF through their paired homeodomains like the MHox gene product.

In the case of the MHox factor, available data suggest that it is able to take part in the control of muscle-specific gene expression in both early myogenic precursors and maturating myofibers. The MHox gene product might act not only by binding to specific DNA sequences but also by interacting with the MADS domain of the SRF. If such an interaction would also occur with the MADS domain of the muscle-specific MEF-2 proteins, one might then expect that the interaction between MADS and paired homeodomain factors might further add to the increasing complexity of the regulatory network controlling myogenesis. While the hypothesis of an interaction between the MHox and MEF-2 factors remains to be tested, the interaction of this paired homeodomain protein with the SRF suggests that it could enhance the activity of SRF target genes in both proliferating myogenic precursor cells and maturating myofibers. Thus the MHox gene product might be involved in the positive control of both myogenic precursor cell proliferation and myofiber maturation through activation of the SRF. As the MHox A+T rich target sequence is also able to bind to the MEF-2 factors, the possibility remains open that MHox competes with MEF-2 for the binding to a subset of MEF-2 sites.

In contrast to our knowledge of gene expression during muscle differentiation, almost nothing is known about the molecular mechanisms which are involved in the control of the very early phase of myogenesis. This analysis is complicated by the fact that further myogenic determination and differentiation occur very shortly after the onset of somitogenesis in the myogenic precursors that will give rise to the trunk muscle. The rapidity of the myogenic process results in apparently simultaneous expression of genes involved in different early processes such as *Pax-3* and *Myf-5*. This apparent overlap might also be due to the lack of resolution at the single cell level of the *in situ* hybridization technology used to describe the expression patterns.

On the other hand the analysis of limb myogenesis might help understanding of the early stages of myogenesis as it occurs much slower than trunk myogenesis. Therefore, we would like to present a model showing the proposed relationships between the various types of myogenic regulatory factors and how they change during mouse embryonic development using limb myogenesis as a biological system. This model is likely to be an oversimplification as the functional interactions between the regulatory factors and the molecular mechanisms involved in their actions are far from being completely understood. It does also not take into account the effects of hormones such as thyroid hormones which positively control muscle differentiation and maturation through their nuclear receptors.

This schematic view of the possible molecular mechanisms involved in the control of limb myogenesis is summarized in Figure 3.

Myogenic limb precursor cells are first detected in the ventrolateral part of the dermomyotome around day 9 of mouse embryonic

development (E9). These cells do not express *Myf-5*, therefore providing evidence for the existence of further upstream early myogenic determination factors (EMDFs), whose identity is not yet known. The identification and characterization of these EMDFs will be a crucial step in our understanding of the early steps of myogenesis. Expression of both the *MHox* and *Pax-3* genes has been demonstrated in these precursor cells. The factors encoded by these genes might be involved in the proliferation of these early myogenic cells, possibly through interactions with the SRF, which might then activate immediate early genes such as *c-fos*, whose protein product is a component of the AP-1 transcription factor which is involved in the control of the expression of growth regulated genes. Subsequently (E9.5), the early myogenic cells are stimulated to migrate toward the limb bud by some signaling molecules which have to be characterized. Analysis of *Splotch* mice has shown that the Pax-3 gene product is required for this migratory process.

As the myogenic cells reach the limb (E10-E10.5) bud they proliferate actively. This proliferative process is likely to be enhanced by the Pax-3 and MHox factors.

At day 10.5 the first *Myf-5* transcripts can be detected in the limbs. Within 24 hours transcripts from the other myogenic HLH factor genes appear. The expression of the *Pax-7* gene has not been documented in limbs before day 14. However, due to its expression pattern in trunk myogenic precursors, we would expect it to be active at these stages. Myf-5, MyoD and Pax-7 gene products are likely to be involved in the onset of a late determination stage which antagonizes cell proliferation and migration which are enhanced by the Pax-3 and MHox factors. This process will result in the downregulation of *Pax-3* and *MHox* transcripts and the arrest of growth stimulation characterized by the disappearance of growth induced factors such as AP-I.

The late determination process ends up with the expression of *myogenin* mRNA and protein. The myogenin factor induces the appearance of MEF-2 factors which cooperate to stimulate an autoregulatory loop. Subsequently, myogenic HLH and MEF-2 factors cooperate to induce transcription of genes encoding muscle-specific markers which accumulate in the newly formed myotubes. The Pax-7 product cooperates with the aforementioned myogenic factors to ensure skeletal myogenic differentiation through stimulation of N-CAM expression and possibly other mechanisms.

At day 13.5 expression of MHox is detected again in the maturating fibers. This factor enhances SRF binding to the CArG motif which is involved in the stimulation of the transcription of a number of muscle-specific genes.

From this model, it appears that, with the exception of the myogenic HLH and MEF-2 factors, little is known from the crosstalks between the various classes of regulators. This might be due to the fact that these factors were isolated by different approaches, thus resulting in different methods of functional analysis. Indeed, while myogenic HLH factors were first isolated on the basis of their ability to convert fibroblast into myoblasts, the SRF, MEF-2 and MHox factors were first characterized as sequence-specific DNA-binding proteins and the *Pax* genes were isolated because of their homology to known *Drosophila* developmental control genes. Thus systematic molecular analysis of these factors and of their functional and possibly molecular interactions will be required to define more precisely their involvement in the molecular control of myogenesis.

These *in vitro* studies must be completed by the analysis of the function of these factors *in vivo* in the embryo. In the cases of *Myf-5*, *MyoD*, *myogenin* and *Pax-3*, mice carrying null alleles are available and have helped understanding of the biological function of the products of these genes. The analysis of *Myf-5* and *MyoD* deficient mice has clearly demonstrated the importance of redundancy mechanisms which can only be revealed when several regulatory genes are inactivated in the same animal.

So far no spontaneous mutations have been described in the *Myf-6*, *Mef-2*, *Mef*-2C, *MHox* or *Pax-7* genes in the mouse system. Therefore, gene-targeting experiments will be required to knock out these genes and analyze their *in vivo* function. Due to possible redundancy mechanisms within each class of regulators and possibly between different types of factors, it may be necessary to generate mice carrying mutations in two or even three regulatory genes. It is not as yet known whether mutations of these genes might be responsible for the generation of human myopathies. Further studies, including comparison of the chromosomal localization of the corresponding human genes (Stapleton *et al.*, 1993) with the increasing number of mapped disease loci will be required to answer this question.

In contrast to the late determination and differentiation stages of the myogenic process, little if any information is available on the possible molecular mechanisms involved in the control of the very early stages of the myogenic determination process. The identification and characterization of the upstream regulators, which control this process, will be a challenging task for the future.

Summary

In the last few years, many aspects of skeletal muscle-specific gene regulation have been explained by the activity of the helix-loop helix (HLH) myogenic regulatory factors of the MyoD family, which are sequentially expressed during skeletal muscle formation. However, evidence is accumulating that muscle specific transcription requires functional interactions of these muscle-specific HLH factors with other regulatory proteins whose expression is not only restricted to skeletal muscle. These regulators include the SRF and MEF2 MADS domain and the MHox paired homeodomain transcription factors. Together with the aforementioned HLH factors, they build an increasingly complex network of regulatory factors. Two members of the Pax multigenic family of developmental control transcription factors, Pax-3 and 7, have been shown to be expressed not only in nervous tissue but also in skeletal muscle precursor cells. Their possible involvement in the control of muscle-specific transcription is discussed in light of known molecular properties of Pax gene products described in other biological systems.

KEY WORDS: *skeletal myogenesis, helix loop helix, MADS domain, paired homeodomain, PAX gene*

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