Pax3 Is Essential for Skeletal Myogenesis and the Expression of Six1 and Eya2*

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Pax3 is a paired box transcription factor expressed during somitogenesis that has been implicated in initiating the expression of the myogenic regulatory factors during myogenesis. We find that Pax3 is necessary and sufficient to induce myogenesis in pluripotent stem cells. Pax3 induced the expression of the transcription factor Six1, its cofactor Eya2, and the transcription factor Mox1 prior to inducing the expression of MyoD and myogenin. Overexpression of a dominant negative Pax3, engineered by fusing the active transcripitional repression domain of mouse EN-2 in place of the Pax3 transcriptional activation domain, completely abolished skeletal myogenesis without inhibiting cardiogenesis. Expression of the dominant negative Pax3 resulted in a loss of expression of Six1, Eya2, and endogenous Pax3 as well as a down-regulation in the expression of Mox1. No effect was found on the expression of Gli2. These results indicate that Pax3 activity is essential for skeletal muscle development, the expression of Six1 and Eya2, and is involved in regulating its own expression. In summary, the combined approach of expressing both a wild type and dominant negative transcription factor in stem cells has identified a cascade of transcriptional events controlled by Pax3 that are necessary and sufficient for skeletal myogenesis.

The final stages of skeletal myogenesis are controlled by a family of basic helix-loop-helix transcription factors, the myogenic regulatory factors (MRFs)

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1 The abbreviations used are: MRFs, myogenic regulatory factors; PCR, polymerase chain reaction; bp, base pairs; kb, kilobase pairs; PBS, phosphate-buffered saline; MyHC, myosin heavy chain.

2 These transcription factors are candidate factors for mediating the response to signals from surrounding tissues and inducing MRF expression. These include Pax3 (17), Six1 (18), Eya2 (19), Mox1/2 (20), and Gli2/3 (21).

Pax3, a member of the paired box family of transcription factors, is first expressed in the paraxial mesoderm and the entire early somite. Subsequently, Pax3 expression is restricted to the developing dermomyotome and maturing cells of the dorso-medial lip, marking the early stages of myogenic cell specification (22, 23). Studies in mice lacking functional Pax3 indicated that Pax3 was required for migrating hypaxial muscle formation (22, 24–26). However, the role of Pax3 in the formation of epaxial muscle was complicated by the presence of Pax7, a closely related member of the Pax transcription factor family (27). Pax7 is able to bind the same DNA motifs as Pax3 (28, 29), and therefore, Pax7 is thought to play a redundant role to Pax3 in the epaxial region of the developing dermomyotome. However, crossing the Myf-5(−/−) mice with the Pax3(−/−) splotch mice (30) identified a requirement for Pax3 in the regulation of MyoD expression, indicating that Pax3 functions upstream of MyoD. The overexpression of Pax3 in paraxial mesoderm leads to activation of muscle-specific gene expression (31, 32).

Recently, the conservation of a developmental pathway utilized in Drosophila for compound eye development has been linked to myogenesis in mammals. In Drosophila, sine oculis (33, 34), eyes absent (35, 36), and dachshund (37) participate in a regulatory cascade initiated by eyeless, a Pax6 homologue (38–40), to regulate compound eye formation. A parallel pathway has been hypothesized to be present during myogenesis involving Pax3 as the initiating factor, and the mammalian homologues of the Drosophila genes as follows: six1, eya2, and dach2 (32, 41, 42). Six1 binds to an essential MEF3 DNA-binding motif in the promoter of the myogenin gene (43). Eya and Dach family members synergize with each other and interact with Six1 to regulate transcription (44, 45). Recently, these molecules were shown to regulate each other’s expression and to induce muscle-specific gene expression in mesoderm explants (32). Furthermore, a Pax3-FKHR fusion protein, which has transforming properties and is present in 60% of all alveolar rhabdomyosarcomas, activates the expression of Six1, MyoD, and myogenin when overexpressed in NIH 3T3 cells (46). However, the relationship of each factor in a regulatory hierarchy, during commitment to the myogenic lineage, is still unclear.

Mox1 and Mox2 are homeobox containing genes that are expressed in overlapping patterns in the paraxial mesoderm (20, 47). During somitogenesis, Mox1 is expressed within the dermomyotome, and Mox2 is expressed in the migrating myoblasts of the limb musculature. Mice lacking Mox2 are defective in limb muscle formation, and the expression of Pax3 and Myf-5 is disturbed in the limb (48). The role of Mox1 during
myotomal muscle formation remains to be determined.

The gli family of zinc finger transcription factors, gli, gli2, and gli3 (21), are the mammalian homologues of the Drosophila cubitus interruptus (Ct), involved in transducing Sonic hedgehog (Shh) signals in responding cells (49). Shh is involved in epaxial muscle determination and activation of Myf-5 expression (50). The role of Gli molecules in Shh signaling and the expression profiles of Gli2 and Gli3 in the developing somite suggest that they are candidates for regulating MRF expression and commitment to the myogenic lineage (51, 52).

A tissue culture system capable of linking somite-patterning events with myogenesis would be valuable for further analysis of the transcription factors involved during myogenic commitment. The P19 cell culture system is such a system because the differentiation of these pluripotent stem cells simulates the biochemical and morphological processes that occur during early embryonic development (53–55). Aggregation of P19 cells in the presence of dimethyl sulfoxide (Me2SO) initiates differentiation into cardiac and/or skeletal muscle depending on the specific serum conditions (56, 57). As P19 cells differentiate into skeletal muscle, they first express markers of the early mesoderm, such as Brachyury T, Wnt3a, and Wnt5b, followed by markers of somitogenesis, such as Pax3, and finally myogenesis markers, such as MyoD and myogenin (58). Thus, differentiating stem cells express the same complement of genes that a mesodermal cell would express as it is induced, forms a somite, and differentiates into myotomal muscle in the embryo.

In this study we utilized the P19 cell culture system in order to examine the events involved in commitment of multipotential muscle precursor cells to the myogenic lineage. We show that during myogenesis in P19 cells, the somite patterning transcription factors, Pax3, Mox1, Gli2, Six1, and Eya2, are expressed prior to MRF expression. Overexpression of Pax3 is sufficient to initiate the proper cascade of events required to induce myogenesis in stem cells. Mox1, Six1, and Eya2, but not Gli2, expression is initiated prior to MRF expression during Pax3-directed myogenesis, indicating that Pax3 expression is sufficient to regulate the expression of these genes. We have also utilized a dominant negative Pax3, created by fusing the mouse EN-2 repression domain to Pax3. We show that Pax3-directed transcriptional activation is essential for proper expression of Six1, Eya2, and Mox1 as well as for myogenesis to occur. However, the presence of the dominant negative Pax3 does not inhibit cardiogenesis, indicating that the requirement of Pax3 during myogenesis is specific for cells committed to skeletal muscle and not all mesoderm cell types.

MATERIALS AND METHODS

Plasmid Constructs—All expression vectors utilized the phosphoglycerate kinase (pgk-1) promoter (59) to drive the expression of various cDNAs. The DNA constructs PGK-Pax3, PGK-Puro, and PGK-LacZ were previously described (58). The DNA construct PGK-Pax3-EN contains the 1.45-kb Pax3-EN-2 fusion cDNA. The 198-amino acid N-terminal repression domain of the mouse EN-2 protein was fused at the C terminus of the first 286 amino acids of Pax3, containing the DNA binding domains. PCR amplification of Pax3 cDNA (17) (GenBank™ accession number NM008781) used the oligonucleotides 5′-AAGACCTCAGGATGGCAGGGTGGCGCTGGCC and 3′-AATCTCGAGTCAATACATTTGAATTTGATATCAATTCGACTTACAGGACCCTGTCGCCC. PCR amplification of EN-2 cDNA (GenBank™ accession number NM010134) used the oligonucleotides 5′-AAGAATTTGACGAAAGGATCTTCAACGGCC and 3′-AAAGATATCTGCCACAGGTGGCCGTGCCT. The 594-bp EcoRV product of the EN-2 PCR product was cloned into the engineered EcoRV site (underlined in the oligonucleotide above) at the 3′ end of the Pax3 promoter alone. PGK vector DNA is a plasmid containing the pgk-1 promoter alone.

Cell Culture and DNA Transfections—P19 embryonal carcinoma cells were cultured as described (55, 57) in 5% Cosmic calf serum (HyClone, Logan, UT) and 5% fetal bovine serum (CanSera, Rexdale, Ontario, Canada). Stable cell lines expressing Pax3 were generated as described previously (58, 60–64). Duplicate transfections using the calcium phosphate method (65) were performed with 8 μg of PGK-Pax3, 0.5 μg of PGK-Puro, 1 μg of PGK-LacZ, and 2.5 μg of B17 (66). P19 control cells were isolated as described (58). Stable cell lines expressing the Pax3-Eng′ fusion were created using the FuGene transfection system according to the manufacturer’s protocol (Roche Molecular Biochemicals). 7 μg of PGK-Pax3-Eng′, 0.5 μg of PGK-LacZ, 0.25 μg of PGK-Puro, and 2.5 μg of B17 were mixed with 17 μl of FuGene reagent and incubated overnight with P19 cells. After 24 h of transfection, efficiency was tested as described (58). 7.5 × 105 cells were plated in a 150-mm dish and selected for puromycin resistance (2 μg/ml). After 10 days, clones were isolated and high expressers identified by RNA slot blots.

Differentiation was induced by aggregating 5 × 105 P19(control), P19(Pax), or P19(Pax3-Eng′) cells in bacterial dishes with or without 0.8% MeSO. The presence of MeSO induces the differentiation of endogenous skeletal and cardiac muscle as described previously in control cells (55, 57, 58). Control cell lines differentiated in the absence of MeSO do not differentiate into cardiac or skeletal muscle. Cells were aggregated for 4 days and then plated into tissue culture dishes and harvested for RNA or fixed for immunofluorescence at the time indicated. MeSO is only added to the media during the aggregation stage of the differentiation. In the time course experiments, 3 × 105 cells were differentiated for day 1 RNA, 2 × 105 cells were differentiated for day 2 RNA, and 5 × 105 cells were differentiated for day 3 RNA. Differentiation was induced by aggregating 5 × 105 cells with 0.8% Me2SO. The presence of Me2SO induces the differentiation of endogenous skeletal and cardiac muscle as described previously in control cells (55, 57, 58). Control cell lines differentiated in the absence of MeSO do not differentiate into cardiac or skeletal muscle. Cells were aggregated for 4 days and then plated into tissue culture dishes and harvested for RNA or fixed for immunofluorescence at the time indicated. MeSO is only added to the media during the aggregation stage of the differentiation. In the time course experiments, 3 × 105 cells were differentiated for day 1 RNA, 2 × 105 cells were differentiated for day 2 RNA, and 5 × 105 cells were differentiated for day 3 RNA.

Northern Blot Analysis—Northern blot analysis was performed as described previously (58). Total RNA (6 μg) was separated on a 1% agarose-formaldehyde gel. Transfer to Hybond-N (Amersham Pharmacia Biotech) occurred by capillary blotting, and RNA was cross-linked by UV irradiation. The membrane was hybridized to DNA probes labeled to over 108 cpm/ml using a random priming labeling kit (Boehringer Mannheim). Coverslips were mounted in a solution of 50% glycerol, 40% PBS, 9.9% para-phenylenediamine and 0.1% Hoechst stain. Immunofluorescence was visualized with a Zeiss Axioskop microscope; images were captured with a Sony 3CCD color video camera and processed using Northern Eclipse, Adobe Photoshop, and CanSera software.

RESULTS

Transcription Factors Found in the Developing Somite Are Expressed Prior to the MRFs during Myogenesis in P19 Cells—We have identified previously the expression patterns of signaling molecules involved in somite patterning during endogenous myogenesis in P19 cells (58). Here we examine the expression pattern, in P19 cells, of transcription factors found...
in the early somite and the maturing dermomyotome during somitogenesis. The expression of myogenin on days 7–9 of the time course indicated the formation of skeletal muscle (Fig. 1A, lanes 8–10). Mox1, a homebox containing transcription factor expressed in the early somite and developing dermomyotome, is expressed from day 3 to day 9, peaking on days 4–6 (Fig. 1B, lanes 4–10). Pax3, Gli2, and Six1 are also expressed during development in the early somite and maturing dermomyotome and were all expressed from day 4 to day 9 (Fig. 1, C–E, respectively, lanes 5–10). Eya2, a cofactor for Six1, found in the embryo only in the developing dermomyotome and not the

FIG. 1. Candidate factors for regulating MRF expression are expressed prior to the MRFs during Me2SO-induced myogenesis in P19 cells. P19 cells were differentiated in the presence of Me2SO to induce skeletal myogenesis by day 9. Northern blots containing 6 μg of total RNA in each lane corresponding to RNA harvested each day during a 9-day differentiation were probed as indicated on the right. Lanes are indicated at the bottom of the figure.

FIG. 2. Pax3 induces myogenesis in pluripotent P19 cells. P19[Pax3] cells (C and D) and P19[Control] cells (A and B) were differentiated in the absence of Me2SO. Cells were fixed on day 9, and immunofluorescence was performed using an anti-MyHC antibody (B and D). Nuclei were stained using Hoechst stain (A and C). As expected for aggregation in the absence of Me2SO, P19[Control] cells did not differentiate into skeletal muscle (B). P19[Pax3] cells differentiated efficiently into skeletal muscle (D). Magnification × 40.

FIG. 3. Pax3 induces the expression of transcription factors that are candidates for regulating MRF expression. Northern blots containing 6 μg of total RNA harvested from P19[Pax3] cells (lanes 1–6) and P19[control] cells (lanes 7–12) on day 0 (lanes 1 and 7) and days 5–9 (lanes 2–6 and 8–12) of differentiation without Me2SO. Blots were probed as indicated on the right and spliced from the same autoradiogram. Control cultures differentiated in the absence of Me2SO did not undergo myogenesis. Lanes are indicated at the bottom of the figure.
levels from day 5 to day 9 (Fig. 3D, lanes 2–6). Six1 and Eya2 were expressed from day 5 to day 9 (Fig. 3, E and F respectively, lanes 2–6). Gli2, which is expressed during Me2SO-induced myogenesis in P19 cells, was not detected by Northern blotting during Pax3-induced myogenesis (data not shown). The timing of the expression of these factors is similar to their expression during myogenesis in parental P19 cells (compare with Fig. 1). Therefore, expression of Pax3 induces the expression of specific factors, Mox1, Six1 and Eya2, prior to the expression of the MRFs leading to myogenesis.

Pax3 Is Essential for Skeletal Myogenesis, but Not Cardiogenesis, in P19 Cells—Since Pax3 was sufficient to induce myogenesis in P19 cells, we undertook a dominant negative approach to determine if it was also essential for myogenesis in P19 cells. To create a dominant negative Pax3, the active repression domain of EN-2 (73, 74) was fused to Pax3 in place of the Pax3 C-terminal activation domain (Fig. 4I). A similar approach has been used by others to block the transcriptional activation of β-catenin (75), Tat (76), and Myb (77). The engrafted repression domain silences transcription through interaction with members of the Groucho/TLE family of transcriptional repressors (reviewed in Ref. 78). We have therefore created a dominant negative Pax3 molecule capable of silencing transcription at promoters bound by Pax3, thereby bypassing the complication of redundancy with Pax7 and/or other Pax family members. This fusion molecule was stably expressed in P19 cells to make P19{Pax3-Eng} cell lines. These cell lines were then differentiated in the presence of Me2SO in order to induce endogenous myogenesis. P19{Control} cells differentiated efficiently into bipolar skeletal myocytes, as shown by the anti-MyHC reactivity (Fig. 4II, B). P19{Pax3-Eng} cell lines aggregated under identical conditions did not differentiate into bipolar skeletal myocytes but did differentiate into cardiomyocytes (Fig. 4II, D) based on a morphological comparison to the bipolar cells in Fig. 4II, B. Furthermore, cardiomyocytes observed under a phase-contrast microscope were rhythmically beating.

To examine the effect of the dominant negative Pax3 molecule on muscle-specific transcripts, Northern blots were examined. The expression profiles of three P19{Pax3-Eng} cell lines were compared with two P19{Control} cell lines (Fig. 5I). The lack of skeletal muscle in P19{Pax3-Eng} cultures in comparison to control cultures was demonstrated by the lack of MyoD...
or myogenin expression (Fig. 5I, A and B, respectively, lanes 6, 8, and 10 compared with lanes 2 and 4). To determine the effect of the dominant negative Pax3 molecule on cardiac muscle-specific transcripts, the transcription factor Gata-4 was analyzed (Fig. 5II). The P19 control cell line and all three P19[Pax3-Eng\(^+\)] cell lines expressed Gata-4 (Fig. 5II, lanes 1–4) indicating that cardiac muscle formed in all of the cultures. This indicates that Pax3 is essential for skeletal myogenesis but that cardiogenesis is unaffected by the loss of Pax3 activity.

**Pax3 Is Essential during Myogenic Commitment**

In this study, we have shown that Pax3 is sufficient to drive skeletal myogenesis when overexpressed in a pluripotent cell line. The differentiation of P19 cells in culture mimics the events that take place during mesoderm induction and myogenesis in the mammalian embryo. The expression of Pax3 in P19 cells induces the expression of other transcription factors present in the developing somite, particularly Mox1, Six1, and Eya2. Furthermore, using a dominant negative Pax3 molecule, we show that Pax3 function is essential for skeletal myogenesis to occur. In addition, Pax3 did not activate, and the dominant negative Pax3 did not inhibit, the cardiac muscle program. The inability to form skeletal muscle due to the loss of Pax3 function is accompanied by the loss of expression of endogenous Pax3, Six1, Eya2, MyoD, and myogenin and a down-regulation of Mox1 expression. Therefore these factors are direct or indirect targets of Pax3 activity. Taking into account the temporal pattern of gene expression observed in experiments of both the overexpression and the loss of Pax3 function, we propose a model in which the signaling cascade involved in fly eye development is conserved in mammalian muscle development as suggested previously (Fig. 7) (41, 42). In this model Pax3 induces the expression of Six1 and Eya2 prior to the expression of MyoD and myogenin. In addition, Pax3 is required for the maintenance but not the initiation of Mox1 expression. In summary, we have further defined the molecular hierarchy surrounding Pax3 function during skeletal myogenesis.

**DISCUSSION**

In this study, we have shown that Pax3 is sufficient to drive skeletal myogenesis when overexpressed in a pluripotent cell line. The differentiation of P19 cells in culture mimics the events that take place during mesoderm induction and myogenesis in the mammalian embryo. The expression of Pax3 in P19 cells induced the expression of other transcription factors present in the developing somite, particularly Mox1, Six1, and Eya2. Furthermore, using a dominant negative Pax3 molecule, we show that Pax3 function is essential for skeletal myogenesis to occur. In addition, Pax3 did not activate, and the dominant negative Pax3 did not inhibit, the cardiac muscle program. The inability to form skeletal muscle due to the loss of Pax3 function is accompanied by the loss of expression of endogenous Pax3, Six1, Eya2, MyoD, and myogenin and a down-regulation of Mox1 expression. Therefore these factors are direct or indirect targets of Pax3 activity. Taking into account the temporal pattern of gene expression observed in experiments of both the overexpression and the loss of Pax3 function, we propose a model in which the signaling cascade involved in fly eye development is conserved in mammalian muscle development as suggested previously (Fig. 7) (41, 42). In this model Pax3 induces the expression of Six1 and Eya2 prior to the expression of MyoD and myogenin. In addition, Pax3 is required for the maintenance but not the initiation of Mox1 expression. In summary, we have further defined the molecular hierarchy surrounding Pax3 function during skeletal myogenesis.
Our finding that Pax3 is sufficient and essential for Six1, Eya2, MyoD, and myogenin expression extends previous findings that Pax3 can induce myogenesis in mesoderm explant experiments (31, 32). In particular, the mesoderm explant studies did not show that Pax3 could activate the expression of Six1, probably due to the high levels of Six1 already expressed in this system. These findings indicated that Pax3 was not upstream of Six1, unlike ey positioning upstream of so during Drosophila development (32, 38). However, our results are consistent with the loss of so and ey expression in Drosophila eyeless (Pax6) mutants (38) and with the conservation of the eye development pathway from the fly being utilized during myogenesis in murine systems.

The observation that the presence of Pax3-Eng abolished the expression of endogenous Pax3 indicates that functional Pax3 is involved in the regulation of its own expression. This finding may define the importance of a previously described regulatory loop in which Six1 and Eya2 activate Pax3 expression in mesoderm explant experiments (32). Since Six1, Eya2, and endogenous Pax3 are down-regulated by Pax3-Eng in our system, our results are consistent with the presence of a Six1-Eya2-Pax3 regulatory loop, regulating Pax3 expression. Alternatively, since the Pax3-Eng protein should silence any promoter containing Pax3-binding sites, it is also possible that Pax3 may autoantigen its own expression, and consequently the Pax3-Eng fusion may inhibit endogenous Pax3 expression.

A role for Mox1 in activating Pax3 expression in the somite remains to be determined, although Mox2 appears to regulate Pax3 expression in the developing limb (48). During endogenous myogenesis in P19 cells, Mox1 is expressed 1 day prior to other transcription factors such as Pax3, Six1, Eya2, and Gli2. This early expression pattern suggests that Mox1 may function upstream of Pax3. In cultures expressing the dominant negative Pax3-Eng fusion, Mox1 expression was down-regulated but not lost. This suggests that Pax3 is involved in the maintenance but not the initiation of Mox1 expression, consistent with Mox1 functioning upstream of Pax3 or in a parallel pathway. Since Mox1 expression is not completely abolished, it is most likely that Pax3 indirectly regulates Mox1 expression rather than binding the Mox1 promoter directly.

We have shown that Gli2 is not a target gene of Pax3 function since Gli2 expression is not observed in differentiated cultures of cells overexpressing Pax3. In addition, Gli2 expression is up-regulated 2-fold in the absence of functional Pax3 suggesting that it may be present in precursors of Pax3-expressing cells. Since endogenous Pax3 expression was not observed, Gli2 is not sufficient to activate Pax3 expression or induce myogenesis in the presence of a dominant negative Pax3 protein. Therefore, if Gli2 plays a role in myogenesis, as studies have indicated (83), it may act in an upstream or a parallel pathway and require the function of Pax3.

The observation that Pax3 is sufficient to induce myogenesis in pluripotent P19 cells indicates that Pax3 is able to commit cells to the myogenic lineage. Recently, Pax7 has been shown to specify the myogenic lineage in muscle stem cells during regeneration and repair of adult muscle (84). Our findings suggest a parallel role for Pax3 during P19 cell myogenesis, which is a model system for myotomal muscle development, compared with Pax7 during the formation of satellite cells. Furthermore, our experiments do not rule out a role for Pax7 in P19 cell myogenesis since Pax3 and Pax7 bind the same DNA element (28). Although our findings in P19 cells remain to be confirmed in the embryo, these studies suggest that Pax transcription factors may play an essential role in specifying the myogenic lineage for muscle that is formed at different times and locations during embryogenesis.

This study has identified the hierarchy of transcriptional events surrounding Pax3 during myogenesis in stem cells. Not only is Pax3 sufficient for myogenesis to take place but it is also necessary for myogenesis. We have further defined the regulatory cascade involving Pax3, Six1, and Eya2 and have shown that Pax3 activity is required to initiate the expression of both Six1 and Eya2 and to maintain high levels of Mox1 expression. Therefore, the order of factors in the cascade involved in Drosophila eye development is conserved in mammalian myogenesis. By directly examining the involvement of Mox1, Six1, and Eya2 in this process, we hope to define the role of each factor in the regulatory cascade controlling myogenesis.

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