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Pax3 modulates expression of the c-Met receptor during limb muscle development

(myogenesis/hepatocyte growth factor/embryogenesis/rhabdomyosarcoma/transcriptional regulation)

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ABSTRACT **Pax3 is a transcription factor whose expression has been used as a marker of myogenic precursor cells arising in the lateral somite destined to migrate to and populate the limb musculature. Accruing evidence indicates that the embryologic origins of axial and appendicular muscles are distinct, and limb muscle abnormalities in both mice and humans harboring Pax3 mutations support this distinction. The mechanisms by which Pax3 affects limb muscle development are unknown. The tyrosine kinase receptor for hepatocyte growth factor/scatter factor encoded by the c-*met* protooncogene is also expressed in limb muscle progenitors and, like *Pax-3*, is required in the mouse for limb muscle development. Here, we show that c-*met* expression is markedly reduced in the lateral dermomyotome of *Splotch* embryos lacking Pax3. We show that Pax3 can stimulate c-*met* expression in cultured cells, and we identify a potential Pax3 binding site in the human c-*MET* promoter that may contribute to direct transcriptional regulation. In addition, we have found that several cell lines derived from patients with rhabdomyosarcomas caused by a t(2;13) chromosomal translocation activating PAX3 express c-*MET*, whereas those rhabdomyosarcoma cell lines examined without the translocation do not. These results are consistent with a model in which Pax3 modulates c-*met* expression in the lateral dermomyotome, a function that is required for the appropriate migration of these myogenic precursors to the limb where the ligand for c-*met* (hepatocyte growth factor/scatter factor) is expressed at high levels.**

Pax3 is a member of a family of developmentally regulated transcription factors critical for proper embryonic development (1, 2). In addition to expression in the central nervous system of the developing embryo, *Pax-3* is expressed early in development in the condensing somite and becomes restricted to the lateral dermomyotome by day 11.5 of mouse embryogenesis (E11.5) (3–7). This region gives rise to the limb musculature. *Splotch* mice deficient for Pax3 fail to develop limb muscles, and human patients with *PAX3* haploinsufficiency can display limb muscle hypoplasia. Pax3 may be important for proper migration of limb myoblasts to the developing limb bud (5–8) and may be required for repression of the myogenic program during this migratory process (9). However, the molecular cascades responsible for these activities remain unknown. Interestingly, in humans a t(2;13) chromosomal translocation juxtaposing the amino terminal DNA binding domains of PAX3 with the transcriptional activation domain of FKHR (a Forkhead family member) results in a fusion protein that is a more potent transcriptional activator than PAX3 itself (10) and causes a tumor of muscle, pediatric alveolar rhabdomyosarcoma (11–13).

Pax proteins are defined by the presence of a 128-aa DNA binding domain termed the paired domain, and some Pax proteins, such as Pax3, also contain a second DNA binding domain, the paired-type homeodomain. The carboxyl termini of these proteins act as transcriptional activation domains (14). Optimized DNA binding sequences recognized by several Pax gene paired domains have been identified (15–17), and the crystal structure of the *Drosophila paired* paired domain bound to DNA has been determined (18). However, with some notable exceptions (19–23), few Pax transcriptional targets have been identified, and no targets for Pax3 have been suggested.

Recent studies have demonstrated that the product of the c-*met* protooncogene is, like Pax3, required for limb muscle development (24). c-*met* encodes the receptor for hepatocyte growth factor/scatter factor (25) and is a member of the receptor tyrosine kinase family (26). An oncogenic translocation involving c-*MET* led to its identification, and c-*MET* is overexpressed in a variety of solid tissue tumors of epithelial origin. Developmental roles for c-*met* in epithelial–mesenchymal interactions and cell motility have also been suggested (27, 28). c-*met* is also expressed in the somite, and homozygous c-*met* knockout mice have no limb muscles (24).

Here, we demonstrate that c-*met* expression is deficient in the lateral dermomyotome of *Splotch* embryos consistent with a function downstream of Pax3. *Pax-3* overexpression in tissue culture cells results in increased c-*met* expression. We identify a consensus Pax3 paired domain DNA binding sequence and show that a potentially functional Pax3 binding site is present in the human c-*MET* promoter. We suggest that Pax3 modulates c-*met* expression during limb muscle development and perhaps in some forms of rhabdomyosarcoma (RMS).

METHODS

Genotyping. Genotyping of *Splotch* embryos was performed by isolating genomic DNA from embryonic membranes of E11.5 embryos and performing PCR with a wild-type or *Splotch*-specific reverse primer and a common forward primer in separate PCRs. (The primer sequences were kindly made available by K. Vogan and P. Gros, McGill University, Montreal). PCR was performed for 30 cycles with an annealing temperature of 65°C and an expected product of ≈1 kb. Primer sequences were as follows: wild-type reverse, 5′-GCGGCTGATAGAACTCACTG-3′; *Splotch* reverse, 5′-GCGGCTGATAGAACTCACAC-3′; forward, 5′-CAGAGACAAATTGCTCAAGGACG-3′.

***In Situ* Hybridization.** *In situ* hybridization was performed as described (29). Washing was performed at 60°C. The c-*met* probe was synthesized using T7 RNA polymerase after subcloning a 267-bp *Bam*HI–*Eco*RI fragment derived from the 5′ end of the murine cDNA (pMMETS; provided by Lloyd Cantley, Harvard Medical School, Boston) into pGEM3Z and

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Abbreviations: GST, glutathione *S*-transferase; RMS, rhabdomyosarcoma; E, embryonic day.

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linearizing with *Hind*III. The *Pax-3* probe was synthesized using T7 RNA polymerase from pBH3.2 (provided by P. Gruss, Max Planck Institute, Gottingen, F.R.G.) after deleting an internal *Sma*I fragment and linearizing with *Hind*III. The *MyoD* probe was synthesized using T7 RNA polymerase from pVCZ11ß after linearizing with *Hind*III (9).

Transfection and Infection of Cultured Cells. These procedures were performed as described (9). Transfections were performed using Transfectam (2.5 μl/μg DNA; Promega) for 6 h. Transiently transfected or infected cells were harvested after 48 h. Stable transfectants were selected at 600 μg of G418 per ml for 2 weeks before cloning. Individual clones were screened for Pax3 expression by Western blot analysis.

Cloning. Cloning of the c-*MET* promoter was performed by amplification of human genomic DNA using primers derived from the published c-*MET* genomic sequence with the addition of restriction sites to facilitate ligation into pXP1 (30), yielding pMET-luc. The amplified product corresponds to 297 bp upstream of the transcriptional start site and the first 22 bp of exon 1. The PCR primers used were: METF, 5′-CG**GGATC**-CCGGGGTGACACTCGCCTCCC-3′; and METR, 5′-CA-CCC**AAGCTT**CGTGTCTGTCTGCCTCGCGTGC-3′ (restriction sites shown in bold face type). A *Bam*HI–*Alu*I fragment containing 87 bp of the 5′ region of this promoter fragment was subcloned into pT81 (30) (containing a minimal thymidine kinase promoter) to yield pT81-MET.

Protocols. Binding site selection assay (17), electrophoretic mobility shift assay (9), and DNase I footprinting (31) protocols have been described. The sequences of the c-*MET* promoter derived oligonucleotides are as follows: MET1, 5′-GGG-GGAGACTCGGTCCCGCTTATCTCCGGCTGTGC-3′; and MET2, 5′-GCAGAGGCGGGAGGAAACGCGACCC-CCGCGGGGCC-3′. The e5 and Nf3′ oligonucleotides have been described (9).

Transactivation Assays. These assays were performed by plating P19 embryocarcinoma cells on 60-mM dishes and transfecting with 0.5 μg pCMV-ßgal, 1 μg reporter plasmid, and 0–5 μg of pcDNA3 control plasmid, pcDNA3-Pax3, or pcDNA3-PAX3/FKHR (9). The total amount of transfected DNA was constant. Luciferase and ß-galactosidase assays were performed according to standard procedures, and luciferase activity was normalized to ß-galactosidase activity.

RESULTS

To test the hypothesis that c-*met* functions downstream of Pax3 during limb muscle development, we examined the expression pattern of c-*met* in Pax3-deficient *Splotch* embryos by *in situ* hybridization. Homozygous Pax3-deficient E11.5 embryos and wild-type littermates were assayed *in situ* for c-*met* expression (Fig. 1). In wild-type embryos, c-*met* is expressed in the ventral neural tube, in the lateral dermomyotome, and in the dorsal and ventral muscle masses of the forelimb (Fig. 1*A*). In Pax3-deficient littermates, c-*met* expression in the dermomyotome is greatly reduced (Fig. 1*B*). (Close inspection of this section and others not shown does reveal low levels of residual expression.) Expression of c-*met* in the dorsal and ventral muscle masses is abolished. Expression in the ventral neural tube persists, as does expression in a localized region of the dorsal forelimb likely representing distal ectoderm. These retained regions of expression serve to demonstrate that the *in situ* hybridization assay was technically successful, and that c-*met* expression outside of the *Pax-3* expression domain is intact.

Adjacent sections of wild-type and Pax3-deficient embryos were analyzed for *Pax-3* transcript expression. The *Pax-3* mutation in *Splotch* is a splice acceptor mutation that results in a stable, nonfunctional transcript (32). *Pax-3* is strongly expressed in the dorsal neural tube and in the dermomyotome. This retained expression of the mutant transcript in the dermomyotome suggests that myogenic precursors that normally express c-*met* are present in the *Splotch* embryos. In control sections, weak *Pax-3* expression is also seen in the dorsal and ventral muscle masses of the forelimb (Fig. 1*C*); this expression domain is absent in *Splotch* embryos (Fig. 1*D*). Strong expression of *MyoD* is noted in the dermomyotome of both wild-type (Fig. 1*E*) and *Splotch* (Fig. 1*F*) embryos, though expression of *MyoD* in the limb bud is absent in *Splotch*, consistent with previous reports (5, 6).

Next, we asked whether Pax3 could upregulate c-*met* expression in cultured cells. C2C12 myoblasts were transiently transfected with *Pax-3* and assayed by RNase protection analysis for *Pax-3* and c-*met* expression. In addition, two transformed NIH 3T3 cell lines (EJ-ras NIH 3T3 and B104 cells) were stably transfected with *Pax-3*, and clonal transfectants expressing high levels of *Pax-3* were isolated. In each case, cells expressing *Pax-3* expressed higher levels of c-*met* than control transfected cells (Fig. 2*A*). We also assayed for c-*met* expression in NIH 3T3 cells infected with control or *Pax-3*-expressing retrovirus, as well as cells infected with virus encoding the PAX3/FKHR fusion protein formed by chromosomal translocation. Both Pax3 and PAX3/FKHR expression resulted in an increased expression of endogenous c-*met* (Fig. 2*B*).

To identify regulatory sequences in the c-*met* promoter potentially responsible for direct Pax3 activation, we determined the consensus binding sequence recognized by the Pax3 paired domain. A PCR-based selection assay beginning with a pool of oligonucleotides containing a core of 25 random nucleotides was used. Sequential rounds of selection with a glutathione *S*-transferase (GST) fusion protein containing the Pax3 paired domain were performed in a manner identical to that used to identify the consensus DNA binding sequences recognized by the Pax2 and Pax6 paired domains (17). After nine rounds of selection, oligonucleotides bound with high affinity were cloned and sequenced and a consensus binding motif was identified (Fig. 3*A*). This sequence is similar to that bound by the amino termini of the paired domains of Pax2 and Pax6 (17) and is similar to the sequence identified by others as a Pax3 paired domain binding site (34). The derived consensus is essentially identical to that reported for the closely related *Drosophila paired* paired domain (18).

Analysis of the published human c-*MET* promoter sequence identifies two potential Pax3 paired domain binding sites based on homology to the consensus binding motif (Fig. 3*B*). Oligonucleotides homologous to these putative binding sites were synthesized and tested in electrophoretic mobility shift assays (Fig. 4*A*). Comparison was made to Pax3 paired domain binding to the e5 sequence [a previously identified Pax paired domain binding sequence derived from the *Drosophila even skipped* gene (35)] and to another previously identified Pax3 paired domain binding sequence (Nf3′) (9) (Fig. 4*A*, lanes 3 and 4 respectively). Binding affinity to the first, more upstream putative site (MET1) was grossly similar to e5 and Nf3′, and a Pro-Leu missense mutation at position 50 of Pax3 within the paired domain [found in some patients with Waardenburg syndrome (36)] abolished binding to this c-*MET* derived oligonucleotide (Fig. 4*A*, lane 5). Only low affinity binding was seen when the more downstream putative site (MET2) was used (Fig. 4*A*, lane 2), consistent with crystallographic evidence for the importance of a C or G at the 14th position of the consensus (as numbered in Fig. 4*A*) which makes contact with a conserved Gly at position 15 of the paired domain (18).

DNase I footprint analysis using the Pax3 paired domain with a 320-bp c-*MET* promoter fragment that is sufficient for inducible c-*MET* expression (33) reveals specific Pax3 paired domain binding to the MET1 region (Fig. 4*B*). This footprint was not seen when a Pax3 paired domain peptide containing the Waardenburg mutation was used (data not shown). No other regions within the proximal *MET* promoter suggestive of Pax3 paired domain binding were identified in this or other

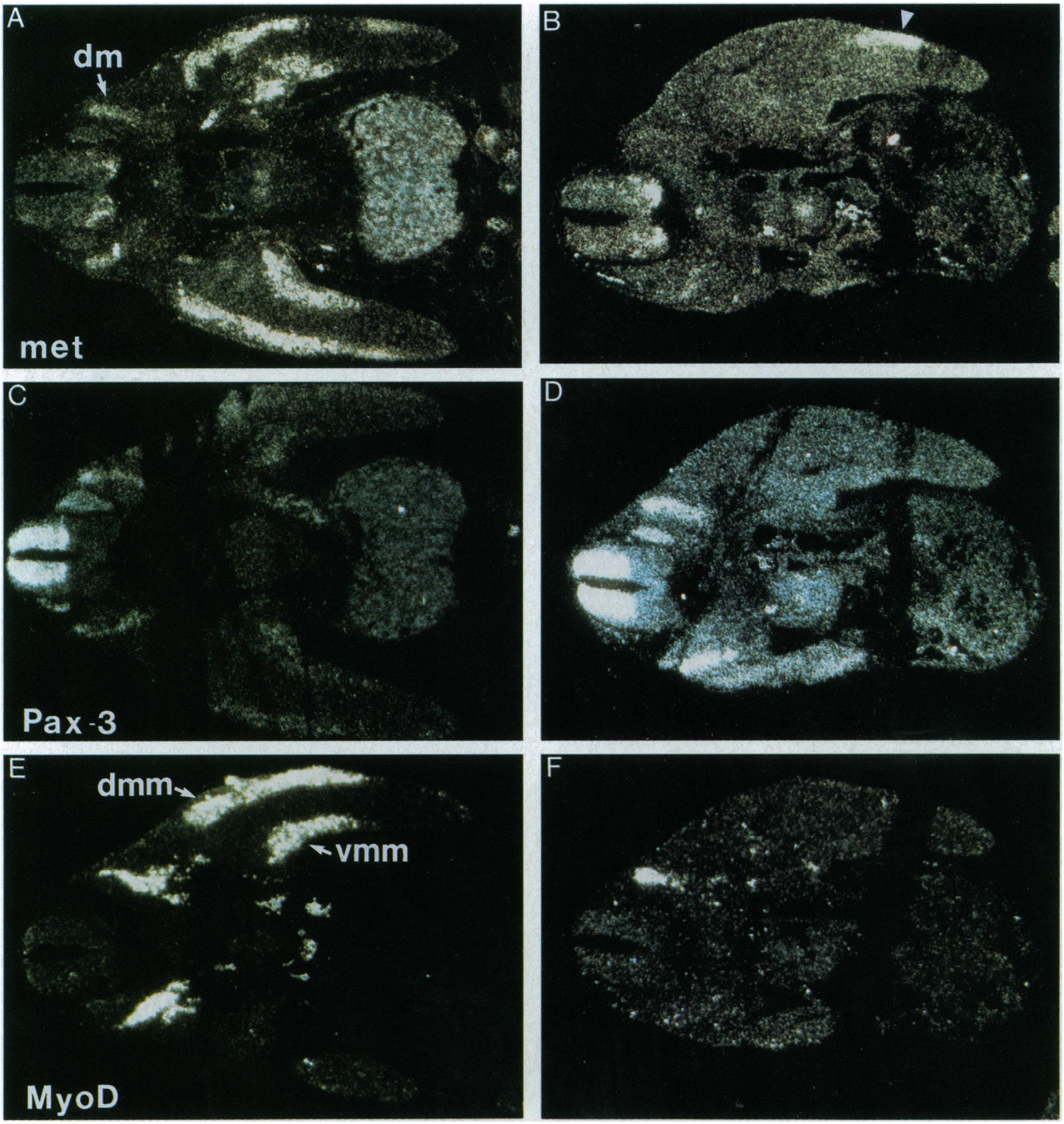

FIG. 1. *In situ* hybridization of E11.5 wild-type (*A, C,* and *E*) and *Splotch* mutant *(Sp/Sp)* embryos (*B, D,* and F) using c-*met* (*A* and *B*), *Pax*-3 (*C* and *D*), and *MyoD* (*E* and *F*) probes. Darkfield images are shown. dm, Dermomyotome; vmm, ventral muscle mass; dmm, dorsal muscle mass. Arrowhead in *B* points to an area of retained c-*met* expression outside of the *MyoD* expression domain, likely representing ectodermal expression. In *C* and *D*, hybridization to the dorsal root ganglia is noted (unlabeled) between the dermomyotome and the neural tube. Staining of the heart is artifactual due to the presence of blood (seen with sense probe, not shown). *B* and *D* are intentionally overexposed to emphasize the absence of signal compared to control.

experiments. Hence, Pax3 is capable of specific binding within the MET1 region of the c-*MET* promoter.

The ability of Pax3 to activate transcription from the c-*MET* promoter was assessed by cloning the proximal c-*MET* promoter upstream of a luciferase reporter gene. P19 cells were cotransfected with the c-*MET*/luciferase reporter and control or PAX3/FKHR expression vectors. PAX3/FKHR was used for these experiments because it is known to be a more potent transcriptional activator than Pax3 (10). Up to a 2.7-fold activation of luciferase activity was seen when PAX3/FKHR was cotransfected with the c-*MET* promoter–reporter construct in a dose-dependent fashion (Fig. 4*C*). The ability of PAX3/FKHR to activate transcription was maintained when a 87-bp c-*MET* promoter fragment containing the MET1 binding site was cloned upstream of a minimal promoter suggesting that this region is sufficient for mediating at least some degree of transactivation by PAX3/FKHR.

Finally, we examined a number of cell lines derived from patients with RMS both with and without the t(2;13) translocation that fuses the DNA binding portion of PAX3 to the

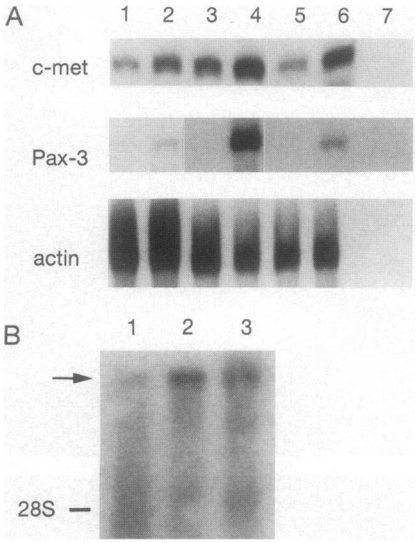

FIG. 2. *(A)* RNase protection assay using 50 μg of total RNA derived from mock-transfected C2C12 cells (lane 1), EJ-NIH 3T3 cells (lane 3), or B104 cells (lane 5), or the corresponding cell lines transfected with *Pax-3* either transiently (C2C12 cells, lane 2) or stably (EJ-NIH 3T3 cells, lane 4; B104 cells, lane 6). Expression of *Pax-3* correlates with increased c-*met* expression. Actin is used as a loading control. *(B)* Northern blot analysis of NIH 3T3 cells infected with control (lane 1), Pax3 (lane 2), or PAX3/FKHR (lane 3) retrovirus. Two micrograms of poly(A)$^+$-selected RNA was loaded per lane. The c-*met*-specific transcript is indicated by the arrow.

transcriptional activation domain of FKHR (Fig. 5). Because PAX3/FKHR can induce c-*met* expression in NIH 3T3 cells, we assayed for endogenous c-*MET* expression in the RMS cell lines. We found that four of five cell lines containing the translocation expressed detectable c-*MET* and that two lines expressed very high levels. All three RMS lines without the translocation failed to express c-*MET* at detectable levels.

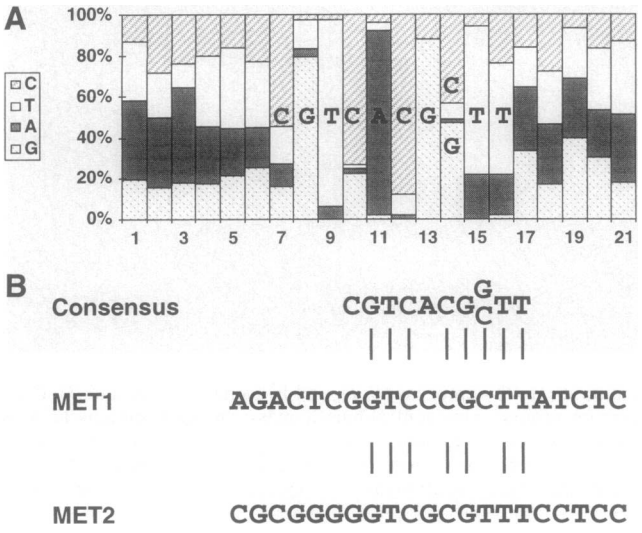

FIG. 3. *(A)* DNA binding site selection assay was performed using a GST–Pax3 paired domain fusion protein and oligonucleotides containing a core of 25 random positions. After nine rounds of selection, 51 selected oligonucleotides were sequenced and aligned using the Genetics Computer Group program PILEUP to yield the consensus binding motif shown. The percent of each nucleotide found at a given position is shown. *(B)* The consensus Pax3 paired domain binding site is aligned with two sequences found in the human c-*MET* promoter (33) identified here as MET1 and MET2. MET1 is located 257 bp upstream of the transcription start site, and MET2 (in the opposite orientation) is 114 bp upstream.

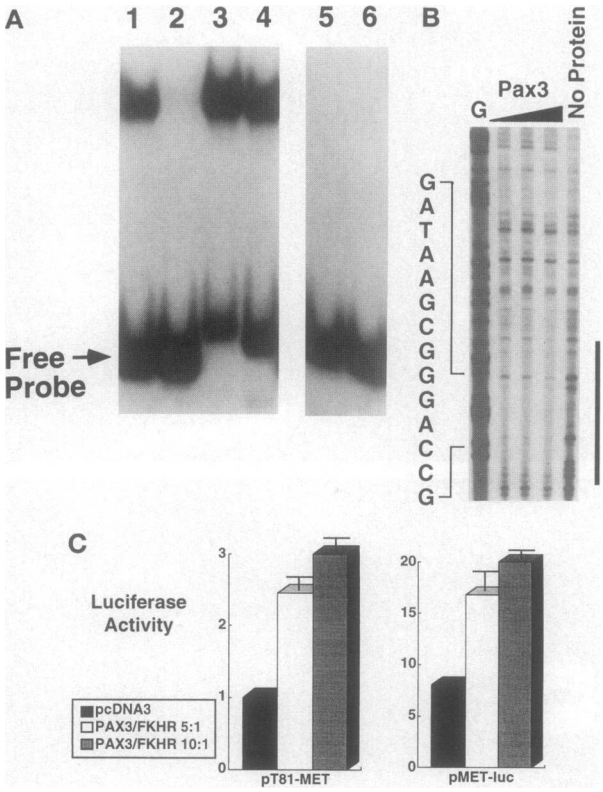

FIG. 4. *(A)* Electrophoretic mobility shift assay using a GST–Pax3 paired domain fusion protein (0.5 μM) and oligonucleotides homologous to regions of the human c-*MET* promoter (MET1, lane 1; MET2, lane 2) and control oligonucleotides known to contain Pax3 binding sites (e5, lane 3; Nf3′, lane 4). A GST–Pax3 paired domain fusion protein containing a point mutation in the paired domain (P50L) found in a patient with Waardenburg syndrome was also used with the c-*MET* promoter oligonucleotides (MET1, lane 5; MET2, lane 6). The Pax3 paired domain bound to MET1, but not MET2, and this binding was abolished by the missense mutation in the paired domain. *(B)* DNase I footprint analysis using the 320-bp proximal c-*MET* promoter and the GST–Pax3 paired domain (0.25–1 μM) reveals binding to the region of the promoter containing the MET1 site. The first lane shows a G ladder of the "bottom" strand, followed by increasing Pax3 paired domain concentrations. The last lane contains no Pax3 protein. The region protected from DNase I digestion is indicated on the right. *(C)* Transactivation of a luciferase reporter gene cloned downstream of the c-*MET* promoter is demonstrated after cotransfection of PAX3/FKHR expression plasmid in 5:1 or 10:1 molar excess. Assays were performed using P19 cells, and luciferase activity is expressed as arbitrary light units normalized for cotransfected β-galactosidase activity. The activity of pT81-MET, containing a minimal promoter only, was assigned a value of 1. Cotransfection of PAX3/FKHR-activated transcription both from the endogenous c-*MET* promoter (pMET-luc), and from an 87-bp promoter fragment containing the MET1 sequence cloned upstream of a minimal heterologous promoter (pT81-MET).

DISCUSSION

In this report, we have identified c-*met* as a target gene regulated by Pax3. Recent work has demonstrated that the myotome can be subdivided into medial and lateral compartments corresponding to progenitors of axial and limb muscle, respectively (37). Formation of axial muscle requires an inductive interaction between somitic cells and axial structures such as the ventral neural tube or the notochord, while formation of appendicular muscle is independent of axial structures (38). Myogenic precursors within the lateral somite are specified but inhibited from myogenic differentiation potentially by signals arising from the dorsal neural tube (39) and/or the lateral plate (40). The lateral plate is capable of inducing *Pax-3*

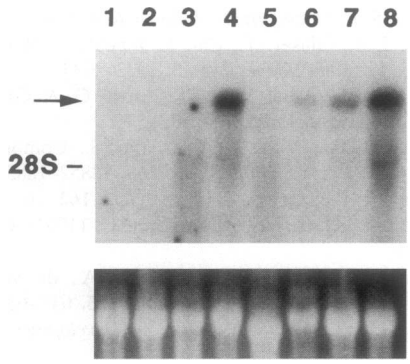

FIG. 5. Northern blot analysis of RNA derived from RMS cell lines without (lanes 1–3) or with (lanes 4–8) the t(2;13) translocation resulting in the expression of the PAX3/FKHR fusion protein. Twenty micrograms of total RNA is loaded per lane, and the blot is probed for c-*MET* expression, seen at the top of the upper panel (arrow). Ethidium bromide staining of the gel is shown below to indicate equal loading of lanes. Four of five cell lines containing the translocation express c-*MET*, while the three cell lines without the translocation do not.

expression in adjacent paraxial mesoderm (40), and Pax3 itself, which becomes restricted to the lateral dermomyotome, may inhibit myogenic differentiation while these cells migrate to the limb (9). The expression domains of *Pax-3* and c-*met* overlap in the lateral dermomyotome and in the limb bud, and both gene products are required for limb muscle development.

We have shown that c-*met* expression in the dermomyotome of *Splotch* embryos is greatly reduced. In a series of *in vitro* systems, Pax3 is capable of inducing c-*met* expression. We suggest that Pax3 in the lateral dermomyotome up regulates c-*met* expression in premigratory myoblasts. Once appropriate c-*met* expression is established, limb myoblast progenitors migrate toward the limb bud where the ligand for c-*met*, the hepatocyte growth factor/scatter factor, is expressed (27). This model accounts for the absence of Pax3-expressing cells in the limb buds of c-*met*-deficient embryos (24). Pax3 may directly activate c-*MET* expression by binding to a motif in the c-*MET* promoter that closely matches an experimentally determined consensus binding sequence recognized by the Pax3 paired domain. Pax3 binding to the proximal c-*MET* promoter is abolished by a missense mutation in the paired domain that causes Waardenburg syndrome.

Our experiments testing the ability of PAX3/FKHR to transactivate a reporter gene downstream of the c-*MET* promoter in cultured cells reveal only modest, albeit dose-dependent and reproducible activation. This degree of activation was maintained when an 87-bp fragment containing the MET1 Pax3 binding site was tested. Experiments using Pax3 instead of PAX3/FKHR showed somewhat less activity (2.4 ± 0.4-fold induction in U20S cells), consistent with the finding that PAX3/FKHR is a more potent transcriptional activator than Pax3 itself (10), although PAX3/FKHR binds with lower affinity to some paired domain binding sites (10, 41). The reasons for the modest responses are unclear. It is possible that other Pax3 regulatory regions are not included in our constructs; identification of these will await more extensive analysis of the c-*MET* promoter. It may also be that P19 cells will not recapitulate the response that occurs in the somite. It is known that P19 cells can be induced to express both *Pax-3* (42) and c-*met* (43) in response to retinoic acid, and hence they seemed reasonable cells to use for these studies. Similar results were obtained in U20S and EJ-NIH 3T3 cells (data not shown). Nevertheless, it is clear that Pax3 will not stimulate c-*met* expression under all circumstances, as is apparent by the strong expression of *Pax-3* in embryonic dorsal neural tube where c-*met* is absent. Perhaps other cofactors are required to see maximal transcriptional activation, as has been shown for the Pbx/Extradenticle family of accessory proteins that cooperatively interact with Hox proteins to modulate DNA recognition and transactivation properties (44).

The phenotypes of *Splotch* and c-*met* knockout embryos are similar only in the common absence of limb musculature, consistent with the overlapping expression of *Pax-3* and c-*met* in this region. *Splotch* embryos also display neural tube and cardiac defects not seen in the c-*met* knockout embryos; clearly, other targets for *Pax-3* exist to account for these abnormalities.

Both c-*met* and *Pax-3* have been implicated not only in limb muscle development but also in oncogenic transformation. The t(2;13) translocation resulting in the PAX3/FKHR fusion protein causes alveolar rhabdomyosarcoma (12, 13), a pediatric tumor of muscle. It is intriguing to hypothesize that the oncogenic activity of this fusion protein may act, at least in part, through a c-*MET*-dependent pathway. We found a striking correlation between the presence of the t(2;13) translocation and detectable c-*MET* expression in RMS cell lines. Nevertheless, since one RMS cell line containing the translocation did not express detectable c-*MET*, activation of c-*MET* alone cannot account for the transformation of these cells, and PAX3/FKHR expression is not sufficient in all cases to activate c-*MET* transcription.

Finally, it is worth noting that other Pax genes and tyrosine kinase receptors may function in related molecular pathways during development, as evidenced by the severe spina bifida defects noted in mice harboring mutations in genes encoding both Pax1 and the platelet-derived growth factor receptor (45). Perhaps one common theme of Pax gene function will prove to be activation of expression of members of the tyrosine kinase family of receptors. In this regard, our preliminary analysis has identified at least one potential Pax paired domain binding site in the *c-kit* genomic sequence.

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