The COOH-terminal Transactivation Domain Plays a
Key Role in Regulating the in Vitro and in Vivo Function of
Pax3 Homeodomain*

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Efficient transcription activation by Pax3 requires binding to a complex DNA sequence element containing binding sites for both the paired domain and the Prd type homeodomain. Previously, we have shown that this requirement is lost in PAX3-FKHR, the product of a t(2;13) chromosomal translocation associated with alveolar rhabdomyosarcoma. In contrast to Pax3, the chimeric PAX3-FKHR, which acts as an oncogene, can efficiently activate a DNA sequence element containing only a homeodomain binding site (TAATAN2–3ATTA), despite the presence of an intact Pax3 paired domain. Here, we showed that this alteration in sequence-specific transcription activity was determined in part by the transactivation domain. First, we demonstrated that in intact Pax3, substitution of the Pax3 transactivation domain with an unrelated viral VP16 transactivation domain enabled Pax3 to transactivate homeodomain-specific DNA sequence, as well as to transform fibroblasts. Furthermore, we could abolish the homeodomain-dependent transcription and transforming activities of PAX3-FKHR by replacing its FKHR transactivation domain with Pax3 transactivation domain. Collectively, these results suggested that the transactivation domain influences the DNA binding specificity of Pax3. The translocation process increased the oncogenic potential of Pax3 by removing the inhibitory action of Pax3 transactivation domain on its homeodomain.

Pax3 is one of the nine-member Pax family of developmentally regulated transcription factors. In murine embryogenesis, Pax3 is first detected in the condensing somite of 8.5-day-old embryo, and later, the expression pattern becomes restricted to primarily the neural crest cell lineage, lateral dermamyotome, and limb bud mesenchyme (1–6). A loss of function in Pax3 has been demonstrated in a mouse Splotch phenotype (7–9) and the human Waardenburg's syndrome types I and III (10, 11). Developmental defects in homozygous Splotch mutants include exencephaly, spina bifida, and deficiency in neural crest derivatives and limb muscle formation. In humans, Waardenburg's syndrome is characterized by defects in pigmentation, craniofacial structure, and limb musculature (specific for type III). In addition to the loss of function mutations, gain of function mutation of Pax3 resulting from chromosomal translocation is associated with alveolar rhabdomyosarcoma.

Rhabdomyosarcoma is a family of soft tissue tumors of skeletal muscle lineage that occurs most frequently in children and young adolescent (12–14). The alveolar subtype represents the most aggressive and malignant form of rhabdomyosarcoma (15–18). A chromosomal translocation involving chromosomes 2 and 13 occurs in more than 90% of alveolar rhabdomyosarcoma (12–14). The two genes involved in the translocation breakpoints have been identified as the Pax3 gene (chromosome 2) and the FKHR gene (chromosome 13) (19–21). Pax3 contains two DNA binding domains: a paired box domain common to all members of the Pax family transcription factors and a Prd-type homeodomain that is also found in Pax4 and Pax6 (22). The Pax3 transcription activation domain is localized within a serine-, glycine-, and threonine-rich region at the COOH terminus. The FKHR gene is related to the developmentally regulated HNF3/forkhead gene family (23–25). This family is characterized by a conserved winged-helix DNA binding domain at the NH2 terminus and a proline-rich acidic transcriptional activation domain at its COOH terminus. The t(2;13) translocation leads to the creation of a PAX3-FKHR chimeric gene that combines the 5’ sequence of the Pax3 gene coding both DNA binding domains of Pax3 to the 3’ sequence of the FKHR gene encoding a partial (bisected) winged helix DNA binding domain and the proline- and acid-rich transactivation domain (19–21).

Although in vivo target genes of Pax3 are currently unknown, Pax3 can bind with high affinity in vitro to a complex e5 DNA response element derived from Drosophila even skipped promoter for the Eve transcription factor (1). This sequence contains a paired domain recognition site GTTCC (PRS-1) adjacent to a homeodomain recognition site (ATTA). The in vitro electrophoresis mobility shift assay and in vivo transfection experiments reveal that both the paired domain and the homeodomain binding sites are required for Pax3 function, suggesting a synergistic interaction between the two DNA domains with the ATTA and GTTCC DNA recognition sites (11, 26). In addition to the DNA binding and transactivation domains, a putative repressor domain has recently been identified within the NH2-terminal 29 amino acid residues overlapping the paired domain of the Pax3, and it functions to inhibit the basal promoter activity in a concentration-dependent manner (27, 28). Because both DNA binding domains of the Pax3 protein are intact whereas the FKHR winged helix DNA binding domain is disrupted in the chimeric PAX3-FKHR protein, it is postulated that Pax3-responsive genes would also be targets for the transcriptional activation of PAX3-FKHR. Indeed, many Pax3-responsive sequences thus far identified also respond to PAX3-FKHR (29). PAX3-FKHR is also a more potent transcription activator than Pax3 (29), leading to the hypothesis that PAX3-FKHR elicits its oncogenic activity by abnor-

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mally up-regulating Pax3-dependent genes. Recently, our laboratory has described a novel mechanism by which Pax3-FKHR gains the ability to transcriptionally regulate DNA sequences that are not normally responsive to Pax3, by recognizing a palindromic paired type homeodomain site from the platelet-derived growth factor-α receptor promoter referred to as p3 (30). Furthermore, mutation of the Pax3 paired domain in Pax3-FKHR has no effect on its transforming activity in NIH3T3 cells, suggesting that homeodomain action alone is sufficient for transforming activity of Pax3-FKHR (31). In the present study, we have analyzed the structure-function requirements for the altered homeodomain DNA recognition specificity between Pax3 and Pax3-FKHR. Our results show that the Pax3 transactivation domain plays a central role in regulating the activity of the Prd type homeodomain.

MATERIALS AND METHODS

DNA Constructs—pCMV expression vector containing the mouse Pax3 and Pax3 mutant cDNAs has been described previously (32). The pDUM expression vector constructs containing the wild type and mutant Pax3-FKHR cDNAs were obtained from J. Epstein (University of Pennsylvania) and have been described previously (29, 33). In brief, the plasmid encoding Pax3-FKHR is a mouse-human chimeric composed of murine Pax3 (GenBankTM accession number X59358, nucleotides 313–298–1420), followed by a linker sequence (GAATTCCGGGATCTGG) and a sequence encoding a nine-residue carboxyl-terminal hemagglutinin epitope tag (YDVPDYASL). The Pax3-FKHR plasmid constructs that contain all three possible ATG translation start codons are referred in this report as the full-length, Pax3-FKHR plasmid constructs that contain all three possible ATG translation and Western blot analysis. Retroviral expression vector containing the mouse Pax3 and Pax3 mutant cDNAs has been described previously (32). The GAL4-VP16 construct was a generous gift from Dr. Michael Green (Massachusetts Medical School). The plasmid construct encoding Pax3/VP16 or Pax3mut/VP16 is composed of murine Pax3 or Pax3mut (GenBankTM accession number X59358, nucleotides 298–1420), followed by a linker sequence (GAATTCCGGGATCTGG) and ending with a VP16 sequence (GenBankTM accession number U89963, nucleotides 418–672). The plasmid construct encoding Pax3-FKHR, the FKHR transactivation domain of which is replaced by the Pax3 transactivation domain, is constructed by first removing the FHKR transactivation domain of Pax3-FKHR (GenBankTM accession number U2368, nucleotides 1833–2559 and the nine amino acid residues of hemagglutinin tag) and then cloning in its place the murine Pax3 transactivation domain (GenBankTM accession number X95358, nucleotides 1334–1737). All expression constructs used in this study were confirmed by sequencing to verify that they expressed correctly sized protein products by a combination of in vitro translation and Western blot analysis. The retroviral expression vector (pBabe-puromycine) was described previously (34). Pax3, Pax3-FKHR, and their derivatives were cloned into the pBabe retroviral expression vector at the BamHI site. Retrovirus producing Phoenix cell line was obtained from Dr. Gary Dolan (University of California). Pax3-specific rabbit polyclonal antibody was a kind gift from Dr. Jonathan Epstein (University of Pennsylvania, Philadelphia, PA). The reporter CAT gene constructs have been described previously (30, 35).

Cell Culture—Monolayer culture of P19 embryonic carcinoma cells were maintained on tissue culture dishes that were pretreated with 0.3% gelatin in the presence of Dulbecco’s modified Eagle’s high glucose medium supplemented with 200 units/ml penicillin, 50 μg/ml streptomycin, 1 mM glutamine, 100 μg/ml sodium pyruvate, and 10% (v/v) bovine calf serum. Murine NIH3T3 cells and Phoenix cells were cultured in the same medium as for P19 but without sodium pyruvate.

Transformation and Infection—Retroviruses expressing Pax3, Pax3-FKHR, or their variant genes were generated by first plating 2 × 106 Phoenix cells onto 60-mm tissue culture dish 24 h prior to transfection. A total of 10 μg of pBabe expression vector (plus or minus the cDNA) was introduced into Phoenix cells by CaPO4 method. After 17 h incubation with DNA, cells were thoroughly rinsed with Tri-S saline and supplemented with fresh growth medium. Cells were allowed to grow for 24 h before the conditioned medium containing retrovirus was filtered and used for infection. Fresh retroviral stocks were used for experiments whenever possible. If stocks were not used within 1 week, they were aliquoted and kept at –80 °C for long term storage.

Twenty-four hours prior to infection, NIH3T3 cells were plated at a density of 2 × 104 cells/100-mm dish. Cells were replenished with fresh medium 4 h before the addition of retrovirus (in the presence of 4 μg/ml polybrene). Cells were allowed to incubate in the presence of virus for a period of 17–24 h before medium change. To establish clonal cell lines, cells were split 1:10 into 150-mm dishes, and single isolates were allowed to form for a period of 14 days in the presence of 2 μg/ml G418. Five to 10 single clones were isolated, expanded, and characterized for protein expression by Western blot analysis before they were used for transformation assays. For each construct, we also expanded and characterized pooled cell populations (>1000 clones) in parallel to verify that results were not due to clonal variability.

Cell Transformation Assays—Transformation of NIH3T3 cells by Pax3, Pax3-FKHR, and their variants was tested using a colony formation in soft agar culture assay (anchorage-independent growth). A total of 1 × 106 cells in 0.3% Noble agar (in growth medium) was laid over a solid support base containing 2% Noble agar (in growth medium). The cells were allowed to grow in suspension for a period of 21–30 days with fresh medium supplementation every 3 days before colonies were counted and photographed.

Transient Transfection and CAT Assays—Transient transfection was carried out by plating P19 cells at a density of 2 × 104 cells/100-mm tissue culture dish 24 h prior to transient transfection. Transient transfection was carried out with a total of 20 μg of DNA that included 1–3 μg of β-galactosidase DNA (LacZ) driven by the β-actin promoter as an internal standard for monitoring transfection efficiency. P19 cells were exposed to DNA-CaPO4 precipitate for 17 h, rinsed, and re-fed with growth medium for an additional 48 h before harvest. Cell lysates for lacZ and CAT assays were prepared as described previously (30). Deacetylase activity in the lysate was inactivated by heating the lysates to 60 °C for 7 min (36). A typical CAT assay reaction mixture consisted of 0.1 μg of acetyl-CoA, 0.2 μCi of [14C]chloramphenicol (1 Ci = 37 GBq), and cell lysates in a final volume of 150 μl. The amount of cell lysate radioactive precipitate was determined for each CAT reaction was standardized by its β-galactosidase activity. Unless stated otherwise, the routine CAT assays were carried out at 37 °C for 1–3 h and terminated by extraction with 1 ml of ice-cold ethyl acetate. Quantitative analysis of CAT activity was carried out by measuring the radioactivity of each radioactive spot in β-scintillation counter.

Western Blot Analysis—To verify proper expression of the different constructs that we made from the Pax3 and Pax3-FKHR constructs, the cDNAs were subjected to in vitro transcription/translation reaction using T7-TNT system (Promega). Sample were analyzed by SDS-polyacrylamide gel electrophoresis and later transferred to nitrocellulose membrane for Western blot analysis. For detecting in vitro expression of Pax3, Pax3-FKHR, and their derivatives, confluent cells were rinsed with cold phosphate-buffered saline twice and lysed in the presence of RNAse precipitation buffer (137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 10% glycerol, 10 μg/ml aprotinin, 500 μM orthovanadate, 1 mM phenylmethylsulfonyl fluoride). Whole cell lysates were fractionated on SDS-polyacrylamide gels and analyzed by Western blot. The expression of Pax3, Pax3-FKHR, or their derivatives was detected by chemiluminescent antibody detection kit (NEF Life Science Products) under the conditions recommended by the manufacturer. The primary rabbit polyclonal Pax3 antibody was used at a dilution of 1:500. The secondary anti-rabbit IgG antibody was used at a dilution of 1:3000.

RESULTS

Previously we have shown that the tumor-specific chimeric protein Pax3-FKHR, but not its normal Pax3 counterpart, can transactivate the P0 homeodomain-specific DNA sequence (TAATN5A) (30). Because both Pax3 and Pax3-FKHR fusion protein contain the same DNA binding domains, we seek to determine how other region(s) of the proteins might influence the selective activity on the homeodomain sequence by Pax3-FKHR but not by Pax3.

The NH2-terminal 10 Amino Acid Residues Are Not Involved in Determining Sequence Specificity of the Prd Type Homeodomain in Pax3 and Pax3-FKHR Proteins—The full-length human and mouse Pax3 cDNAs contain three in-frame ATG codons. It is presently unknown which of the three ATG codons

\(^1\) The abbreviation used is: CAT, chloramphenicol acetyltransferase.
(any one or two or all three) are used for in vivo expression of Pax3 and PAX3-FKHR in development and in rhabdomyosarcoma formation. Constructs that began from the second ATG were referred to as truncated and are denoted with the letter t. B, schematic illustration of the components in the complex 6e5-ECCAT and simple P3-TKCAT reporter constructs. The 6e5-ECCAT contains six tandem copies of the complex e5 sequence (TCGGGCACGCGAGCTGGATAGCACCCTCCGCTCAAGGCTCGG) inserted upstream of the E1B-TATA basal promoter driving the CAT reporter gene. The P3-TKCAT construct contains a single copy of the palindromic Prd type homeodomain recognition sequence, CAGTTTTCTAAATCCCATTAAAAGGATTAGCAACTAC, inserted upstream of the thymidine kinase basal promoter driving the CAT reporter gene. C, P19 cells were transiently transfected with a total of 20 μg of DNA (10 μg of the either ECCAT or 6e5-ECCAT reporter construct, 0.1 μg of the pcDNA3 empty vector or vector containing Pax3 cDNA, 3 μg of β-galactosidase gene under the control of β-actin promoter, and nonspecific pGEM3 plasmid DNA) under the conditions described under “Materials and Methods.” Whole cell lysates were prepared 48 h after transfection and assayed for both β-galactosidase activity and CAT activity. The amount of lysates used for CAT assays were normalized to the β-galactosidase activity. D, P19 cells were transiently transfected with a total of 20 μg of DNA (2.5 μg of either TKCAT or P3-TKCAT, 1 μg of the pcDNA3 empty vector or vector containing Pax3 cDNA, 3 μg of β-galactosidase gene under the control of β-actin promoter, and nonspecific pGEM3 plasmid DNA) under the conditions described under “Materials and Methods.” The mean fold induction was calculated over the CAT activity measured in cells co-transfected with the empty pcDNA3 expression vector, which was assigned an arbitrary value of 100%. Values shown represent means of a minimum of three experiments. The S.D. is defined as the root mean square deviation of n – 1 determinations.

FIG. 1. Effect of the NH2-terminal 10 amino acid residues on Pax3 activity. A, schematic illustration of the Pax3 expression constructs with or without the NH2-terminal 10 amino acid residues. Met1 (M1) and Met11 (M11) are methionine residues encoded by the first and second ATG codons in the full-length Pax3 cDNA, respectively. Constructs that began from the second ATG were referred to as truncated and are denoted with the letter t. B, schematic illustration of the components in the complex 6e5-ECCAT and simple P3-TKCAT reporter constructs. The 6e5-ECCAT contains six tandem copies of the complex e5 sequence (TCGGGCACGCGAGCTGGATAGCACCCTCCGCTCAAGGCTCGG) inserted upstream of the E1B-TATA basal promoter driving the CAT reporter gene. The P3-TKCAT construct contains a single copy of the palindromic Prd type homeodomain recognition sequence, CAGTTTTCTAAATCCCATTAAAAGGATTAGCAACTAC, inserted upstream of the thymidine kinase basal promoter driving the CAT reporter gene. C, P19 cells were transiently transfected with a total of 20 μg of DNA (10 μg of the either ECCAT or 6e5-ECCAT reporter construct, 0.1 μg of the pcDNA3 empty vector or vector containing Pax3 cDNA, 3 μg of β-galactosidase gene under the control of β-actin promoter, and nonspecific pGEM3 plasmid DNA) under the conditions described under “Materials and Methods.” Whole cell lysates were prepared 48 h after transfection and assayed for both β-galactosidase activity and CAT activity. The amount of lysates used for CAT assays were normalized to the β-galactosidase activity. D, P19 cells were transiently transfected with a total of 20 μg of DNA (2.5 μg of either TKCAT or P3-TKCAT, 1 μg of the pcDNA3 empty vector or vector containing Pax3 cDNA, 3 μg of β-galactosidase gene under the control of β-actin promoter, and nonspecific pGEM3 plasmid DNA) under the conditions described under “Materials and Methods.” The mean fold induction was calculated over the CAT activity measured in cells co-transfected with the empty pcDNA3 expression vector, which was assigned an arbitrary value of 100%. Values shown represent means of a minimum of three experiments. The S.D. is defined as the root mean square deviation of n – 1 determinations.

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CAT construct. Mutation of the Pax3 paired domain (Pax3mut-Bu26, tPax3mut-Bu35, and tPax3mut-Un1) abolished transactivation by both full-length Pax3 and tPax3. The Bu26 (P50L), Bu35 (R56L), and UN1 (G48S) mutations represent naturally occurring missense mutations found of the paired domain of Pax3 (4), and all three mutations have previously been shown to abrogate the DNA binding activity of Pax3 and PAX3-FKHR toward the complex e5 DNA sequence. Under the same assay conditions, neither Pax3 nor tPax3 could transactivate the P3-TKCAT construct (Fig. 1D).

In a second approach, we reintroduced the sequences encoding the NH2-terminal 10 amino acids back into tPF (Fig. 2A) and examined whether these residues could block the independent homeodomain specificity of the full-length PAX3-FKHR (referred to here as PF). As could be predicted from results in Fig. 1, both wild type PF and tPF transactivated the 6e5-ECCAT construct, whereas their corresponding paired domain mutants (PF-Bu26 and tPF-Bu35) were inactive (Fig. 2B). Furthermore, both PF and tPF, as well as their corresponding paired domain mutants, remained transcriptionally active toward the P3-TKCAT construct (Fig. 2C). The observed transactivation was clearly mediated through a specific interaction between the homeodomain region of the proteins and the homeodomain-specific P3 sequence because mutation of the homeodomain region in these proteins (PF-HD and tHD) or mutation of the P3 consensus sequence (mP3) completely abolished the transactivation activity of PF and tPF. It should be noted that although there was no difference in the specificity of PF and tPF to transactivate reporter construct containing either the complex e5 or homeodomain-specific P3 response ele-

FIG. 2 Effect of NH2-terminal 10 amino acid residues on PAX3-FKHR fusion protein activity. A, schematic illustration of the PAX3-FKHR expression constructs with or without the NH2-terminal 10 amino acid residues. Met1 (M1) and Met11 (M11) are methionine residues encoded by the first and second ATG codon in the full-length PAX3-FKHR cDNA, respectively. Constructs that began from the second ATG were referred to as truncated and are denoted with the letter t. B, transactivation of ECCAT or 6e5-ECCAT reporter gene by wild type and mutant PAX3-FKHR with or without the NH2-terminal 10 amino acid residues. C, transactivation of TKCAT, P3-TKCAT, or mP3-TKCAT reporter gene by wild type and mutant PAX3-FKHR with or without the NH2-terminal 10 amino acid residues. The mP3 was a mutant of P3, the TAACTCCATTA sequence of which was changed to GCCGCCGGGC. Conditions used for co-transfection for both B and C were the same as those described in the Fig. 1 legend. The mean fold induction was calculated over the CAT activity measured in cells co-transfected with the empty pcDNA3 expression vector, which was assigned an arbitrary value of 100%. Values shown represent means of a minimum of three experiments. The S.D. is defined as the root mean square deviation of n – 1 determinations.
ment, we did observe a significant increase in background activity by the full-length PAX3-FKHR. This increase in background activity was not observed with the Pax3 construct (see under "Discussion").

Taken together, the data from Figs. 1 and 2 indicated that NH2-terminal 10 amino acid residues of Pax3 did not play a role in controlling the transcriptional potency or in the homeodomain specificity of both Pax3 and PAX3-FKHR proteins.

The Pax3 Transactivation Domain Negatively Regulates the Functional Activity of the Homeodomain—We next tested the possibility that DNA sequences downstream of the Pax3 DNA binding domain may mediate the difference between Pax3 and PAX3-FKHR in transactivation of the P3-TKCAT construct. To begin addressing this question, we deleted intervening sequences between the DNA binding domains and the COOH-terminal transactivation domain from Pax3 and from Pax3mut-Bu26, a paired domain mutant (Fig. 3A). In addition, we also substituted the viral VP16 transactivation domain for the Pax3 transactivation domain in Pax3 and Pax3mut-Bu26 (Fig. 3A). Each of these constructs was co-transfected into P19 cells with either the 6e5-ECCAT (Fig. 3B) or P3-TKCAT (Fig. 3C) reporter gene constructs. As shown in Fig. 3B, neither the intervening sequence nor the Pax3 transactivation domain was required to transactivate the complex e5 containing reporter gene construct. As expected, none of the paired domain mutant expression vectors could transactivate the complex e5 sequence. Strikingly, substitution of the VP16 transactivation domain for the Pax3 transactivation domain enabled Pax3 to transactivate the P3-TKCAT construct (Fig. 3C). This transactivation was homeodomain-specific because mutation of the paired domain (Pax3mut/VP16) did not affect transactivation of the P3-TKCAT construct. The internal deletion mutants of Pax3 (Pax3del8 and Pax3mutdel8) remained unable to transactivate the homeodomain-specific P3 DNA sequence. Collectively, these data led us to hypothesize that the COOH-terminal Pax3 transactivation domain played a decisive role in blocking Pax3 action toward homeodomain. If so, substitution of the FKHR transactivation domain by Pax3 transactivation domain in PAX3-FKHR would relieve the negative constraint imposed on the homeodomain by Pax3 transactivation domain,
allowing it to function as an independent DNA binding domain in the fusion protein.

To test this hypothesis, we replaced the FKHR transactivation domain with the Pax3 transactivation domain in wild type and paired domain mutant of PF and tPF (Fig. 4A). When these constructs were co-transfected into cells with the 6e5ECCAT, they were able to transactivate the complex 6e5 sequence (Fig. 4B). As expected, these constructs containing the Pax3 transactivation domain showed a lower fold induction than the constructs containing the FKHR transactivation domain. Most interestingly, replacement of the FKHR transactivation domain with the Pax3 transactivation domain in PF and tPF and their paired domain mutants (PF-Bu26/Pax3Td and PF-Bu26/Pax3Td) rendered the proteins inactive toward the P3-TKCAT construct (Fig. 4C). The paired domain deletion mutant of PAX3-FKHR (tPDNH2) contains a deletion of the first three helices of paired domain (Asn53 to Thr93) that is important for DNA contact. As expected, the paired domain mutants were also inactive.

The Pax3 Transactivation Domain Blocks the in Vivo Oncogenic Property of PAX3-FKHR—Thus far, we have presented evidence from the transfection assay to suggest that the
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Fig. 5. Effect of Pax3 transactivation domain substitution on the transforming activity of Pax3 and PAX3-FKHR in NIH3T3 cells. A, Western blot analysis of in vivo expressed Pax3, PAX3-FKHR, and their variants in NIH3T3 cells. A total of 20 μg of whole cell lysate was used for the analysis under the conditions described under "Materials and Methods." B, anchorage-independent growth assay was carried out by overlaying 1×10^6 cells in 0.3% Noble agar (in growth media) and over that, a solid support base containing 2% Noble agar (in growth media). The cells were allowed to grow in suspension for a period of 21 days. Cells were replenished with fresh media in the presence of 2 μg/ml puromycin every 3 days before photography.

COOH-terminal transactivation domain of Pax3 played a key role in the sequence specificity of transactivation. Could our observation be correlated to the biological activity of the PAX3-FKHR fusion protein? We examined this issue by testing the effect of Pax3 transactivation domain substitution on the ability of PAX3-FKHR to induce cell transformation (Fig. 5). We used the retroviral expression system to induce stable expression of PAX3-FKHR and its derivatives in NIH-3T3 fibroblast cells (Fig. 5A) and tested the anchorage-independent growth property of these cells in soft agar (Fig. 5B) and tested the anchorage-independent growth property of these cells in soft agar. Mutation of the PAX3-FKHR homeodomain (tPF-HD) abolished the transformation activity of the fusion protein. A similar result was obtained when full-length PAX3-FKHR was tested (data not shown). These results were in agreement with the previous findings by Lam et al. (31). NIH3T3 cells expressing wild type (PF/Pax3Td and tPF/Pax3Td) and paired domain mutant (PF-Bu26/Pax3Td and tPD/Pax3Td) PAX3-FKHR, the FKHR transactivation domains of which were replaced by the Pax3 transactivation domains, were unable to grow in anchorage-independent fashion. Thus replacement of the FKHR transactivation domain in PAX3-FKHR with the Pax3 transactivation domain abolished the transforming activity of PAX3-FKHR. This was not due to a specific function missing from the FKHR transactivation domain because substitution of the viral VP16 transactivation domain into Pax3 (Pax3/VP16 and Pax3mut/VP16) was sufficient to allow Pax3 to transform NIH3T3 cells. The result in Fig. 5 established a complete correlation between the homeodomain-mediated transactivation activity and the oncogenic property of PAX3-FKHR.

DISCUSSION

The t(2;13) chromosomal translocation that joins the NH2-terminal region of the Pax3 gene with the COOH-terminal region of the FKHR gene is implicated in the development of alveolar rhabdomyosarcoma. The resulting chimeric transcription factor PAX3-FKHR has a gain of function phenotype in that it can transform fibroblast cells in culture, i.e. it can act as an oncogene. The mechanism underlying this gain of biological function has not yet been elucidated. Previous studies have shown that PAX3-FKHR demonstrates an increased transcription activity on Pax3-responsive DNA sequences. The increased transcription potency in PAX3-FKHR has been explained in part to result from the switch of transactivation domains. It has been suggested that the increased activity of the chimeric PAX3-FKHR results from the inability of the FKHR transactivation domain to be inhibited by either a NH2-terminal cis acting inhibitory domain (27, 28) or by a trans-acting repressor Daxx protein (39). One mechanism for the oncogenic activity of PAX3-FKHR may be mediated by this increased transactivation activity that can abnormally regulate expression of Pax3 target genes, causing disruption in process involving myoblast cell proliferation, differentiation, or migration.

Recently, we have proposed an alternative mechanism for the transforming activity of PAX3-FKHR that is based on the observation that PAX3-FKHR can increase transcription of genes that are not normally targets of Pax3 or FKHR action (30). Several studies have shown that Pax3 protein contains two structurally distinct DNA binding domains, a paired domain and a Prd type homeodomain. Both protein-DNA binding and transcriptional assays have revealed that in Pax3, the two DNA binding domains function interdependently. The homeodomain functions to facilitate efficient paired domain DNA binding activity, and the paired domain regulates the DNA binding specificity and dimerization potential of the homeodomain. Thus, transcriptional activation by Pax3 requires binding to a composite site containing both a paired domain and a homeodomain-specific sequence. Consistent with this notion, mutation of either DNA binding domain in Pax3 abolishes its transcription activity. By contrast, we have demonstrated that in addition to Pax3-dependent DNA sequences, the chimeric PAX3-FKHR protein shows an altered sequence recognition specificity and is able to bind and activate DNA sequences containing either Pax3 or Pax3mut-Bu26 cDNAs failed to form colonies in soft agar, whereas NIH3T3 cells expressing wild type tPF and paired domain mutant tPF-Bu35 were able to form large colonies in soft agar. Mutation of the PAX3-FKHR homeodomain (tPF-HD) abolished the transformation activity of the fusion protein. A similar result was obtained when full-length PAX3-FKHR was tested (data not shown). These results were in agreement with the previous findings by Lam et al. (31). NIH3T3 cells expressing wild type (PF/Pax3Td and tPF/Pax3Td) and paired domain mutant (PF-Bu26/Pax3Td and tPD/Pax3Td) PAX3-FKHR, the FKHR transactivation domains of which were replaced by the Pax3 transactivation domains, were unable to grow in anchorage-independent fashion. Thus replacement of the FKHR transactivation domain in PAX3-FKHR with the Pax3 transactivation domain abolished the transforming activity of PAX3-FKHR. This was not due to a specific function missing from the FKHR transactivation domain because substitution of the viral VP16 transactivation domain into Pax3 (Pax3/VP16 and Pax3mut/VP16) was sufficient to allow Pax3 to transform NIH3T3 cells. The result in Fig. 5 established a complete correlation between the homeodomain-mediated transactivation activity and the oncogenic property of PAX3-FKHR.
containing a palindromic Prd type homeodomain element. Thus, we hypothesized that the homeodomain-mediated PAX3-FKHR activity may be important for the oncogenic potential of PAX3-FKHR. It is possible that the fusion process induces a conformational change within PAX3-FKHR, enabling it to regulate the expression and function of additional set of genes that are not normally regulated by either Pax3 or FKHR. One prediction of this hypothesis would be that only the homeodomain DNA binding motif of PAX3-FKHR could be required for transformation. Recently, Lam et al. (31) have shown that mutation of the paired domain of PAX3-FKHR has no effect on the transforming activity of the fusion protein. In this study, we carried out deletion mapping and domain swapping experiments to determine the region(s) of PAX3-FKHR protein responsible for the altered DNA recognition specificity.

One of the points that we focused on was the NH2-terminal 10 amino acid residues of the Pax3 protein. There are three in-frame ATG codons encoding methionine residues at amino acid positions 1, 10, and 11 of the full-length Pax3 protein. It is currently not known whether all three codons are used by Pax3 or PAX3-FKHR during development and in rhabdomyosarcoma formation. If all three codons are used in vivo, they will generate protein species that differ in 10 or 11 amino acid residues at the NH2 terminus. Our current data demonstrate that the full-length PAX3-FKHR cDNA displays the same sequence-specific transcriptional activity (Fig. 2) as does the NH2-terminally truncated PAX3-FKHR cDNA that begins from the sec-

In conclusion, we have demonstrated in this study that the in vitro protein-DNA binding assays have shown that the homeodomain, when expressed as a separate entity, can bind and dimerize on the homeodomain-specific P3 palindromic recognition motif. However, it fails to dimerize on P3 sequence when the homeodomain is expressed together with the paired domain in the same polypeptide (40). Although the second helix of the paired domain has recently been shown to play a major inhibitory role to the in vitro binding specificity of homeodomain on P3 sequence (41), we show by functional analysis that absence of the second helix (e.g. the PDPax3/HD construct) is insufficient to uncouple regulation of homeodomain by the paired domain (Fig. 4). It will be of interest to find out whether removal of one or more of the remaining three helices of the paired domain would be sufficient to uncouple regulation of homeodomain function by the paired domain.

At present, it is possible to suggest that in intact Pax3 protein, the Pax3 transactivation domain may interact directly either with both DNA binding domains to bring them into their correct structural configuration or primarily with the homeodomain, thus blocking it from functioning alone. The introduction of FKHR transactivation domain as a result of t(2;13) translocation could significantly disrupt interactions within DNA binding domains and the transactivation domain, allowing the homeodomain to function as an independent entity. Alternatively, it is possible that Pax3 and FKHR transactivation domains interact differently with another protein factor(s) that in turn directly interacts with and influences the homeodomain DNA binding specificity. Recently, it has been reported that a repressor protein named Daxx binds with equal efficiency to Pax3 and PAX3-FKHR, primarily through their homeodomain binding domains (39). It will be interesting to know whether Daxx or other unknown protein factors participate in determining the DNA binding specificity of the Pax3 homeodomain.

In conclusion, we have demonstrated in this study that the specificity of Prd type homeodomain of Pax3 is tightly regulated by transactivation domain present in the full-length protein. Further studies are needed to better understand how the Pax3 and FKHR transactivation domains communicate with the Pax3 DNA binding domains. Because homeodomain-mediated activity is unique to the tumor-specific PAX3-FKHR fusion protein, understanding the molecular basis for altered PAX3-FKHR binding specificity may allow development of an effective drug therapy to disrupt its oncogenic function with little or no effect on normal gene function.

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