Pax-3 contains domains for transcription activation and transcription inhibition
(homeobox/paired box/DNA binding)

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ABSTRACT Pax-3 is a member of the Pax family of transcription factors involved in transcriptional control events during embryonic development. Here we report a functional dissection of the Pax-3 protein and describe the protein domains which are responsible for different activities. A transcription inhibition activity is located in the first 90 N-terminal amino acids and includes part of the paired domain. Furthermore, the C terminus of Pax-3 is able to confer transcriptional activation of basal promoters. Pax-3 can utilize both transcription modulating functions and activates transcription over a narrow range of protein concentration in the presence of promoter elements containing functional binding sites.

Pax-3 is expressed early during embryonic development in spatially restricted domains in the nervous system and in some mesodermally derived structures (1). The expression pattern of Pax-3 suggests that it plays an important role in the regional specification of certain aspects of the nervous system. Recently, this hypothesis has been confirmed as mutations in the Pax-3 gene were found to be responsible for the splotch (sp) phenotype in mice (2-5) and the Waardenburg syndrome type 1 (WS1) in humans (6-10). Furthermore, a chromosomal translocation of Pax3 (the human homolog of mouse Pax-3; ref. 11) is implicated in the generation of the pediatric solid tumor alveolar rhabdomyosarcoma (12, 13). Finally, several Pax gene products, including Pax-3, have been shown to induce transformation of cells in culture, and these Pax-transformed cells can subsequently induce tumors in recipient mice (14).

The Pax-3 protein (479 amino acids long) contains two different DNA-binding domains, a paired domain (PD), and a paired-type homeodomain (HD). An octapeptide sequence motif that is conserved among most PD-containing proteins is located between the PD and the HD. Pax-3 binds in vitro to a DNA sequence derived from the Drosophila even skipped promoter (1, 15). This sequence contains an upstream ATTA motif recognized by the HD and a downstream paired domain recognition site (PRS), either GTTCC (PRS-1) or GTTAC (PRS-9), bound by the PD (15). Efficient binding of Pax-3 to the PRS sequences requires the presence of both DNA recognition motifs (ATTA and either GTTCC or GTTAC), suggesting a synergetic interaction of the two DNA-binding domains with the PRS sequences. The lengths of the spacer sequence between the ATTA and GTTCC motifs can vary considerably without affecting the binding of Pax-3 (16).

In addition, the space between the PD and the HD (53 amino acids) can be shortened without changing the flexibility of Pax-3 to interact with the PRS sequences (16). Finally, the protein region between the PD and the HD was found to participate in the homodimerization of truncated Pax-3 protein products when bound to the DNA (15). In this work, we describe the dissection of the Pax-3 protein functions and ascribe specific functional activities, such as transcription inhibition and activation, to discrete domains of Pax-3. When PRSs are inserted in front of a minimal promoter, Pax-3 can utilize these sequences to modulate the transcription rate of a linked reporter gene. Lower Pax-3 concentrations activate transcription, whereas higher concentrations inhibit the basal promoter activity in cell culture experiments. These data suggest that Pax-3 may be a bifunctional transcriptional regulatory protein.

MATERIALS AND METHODS

Recombinant Plasmids. The plasmids RF1-Pax3 and pS1-Pax3 (15) were the starting plasmids used to generate all Pax-3 deletion mutants. Numbers in the parentheses refer to the nucleotide positions on the Pax-3 cDNA (1). The HindII(408)-XbaI, StuI(442)-XbaI, and NcoI(534)-XbaI fragments were blunt-ended and subcloned into the SacI (blunt-ended with T4 DNA polymerase) site of pS1-Pax3. The XbaI site of the fragments was always cloned into the XbaI site of the vector. The BamHI-HindIII fragments of these constructs were then subcloned into the BamHI-HindIII sites of RF1-Pax3 to create ΔHind, ΔStu, and ΔNco. BamHI and ClaI (858) restriction digestion of RF1-Pax3, treatment with the Klenow fragment of DNA polymerase I, and religation led to Δ-Cla. Δ-Sma lacks the internal SmaI fragment (342-673). The BamHI-PvuII (985) and BamHI-PvuII (1213) fragments from RF1-Pax3 were subcloned into the BamHI/Smal sites of pS1 and the resulting BamHI-XbaI fragments were introduced into the BamHI/XbaI sites of pEVR1 to generate Pvu1-Δ and Pvu2-Δ. The BamHI-PvuII (1578) fragment from RF1-Pax3 was subcloned into the BamHI/Smal I sites of pS1. The Pax-3 cDNA fragment was then excised by digestion with BamHI and DraI and subcloned into the BamHI/PvuII (1844) sites of RF1-Pax3 to generate Pvu3-Δ. A 33-bp DNA fragment from the polylinker region of pS1 (5′-CGATCCGTCGACGACCCCCGAGACATCGAG3′) was inserted into the HindIII(1065) site in RF1-Pax3 to introduce 11 amino acids (Leu-Ile-Arg-Arg-Tyr-Pro-Gly-Glu-Asp-Glu) between the lyssine and leucine in the turn of the helix-turn-helix motif of the Pax-3 HD and to create Pax3-MHD. Pax3-Moct was constructed by introducing 9 bp into the ClaI (858) site of RF1-Pax3 (5′-CGAATTCGCCCCGAGACATCGAG3′), which then introduces three additional amino acids (Glu-Pro-Pro) between the isoleucine and aspartic acid in the Pax-3 octapeptide. The reporter plasmid 17M2-TK-CAT was kindly provided by P. Chambon (17) and the GAL4 expression vector pSG424 was provided by I.

Abbreviations: PD, paired domain; HD, homeodomain; PRS, paired domain recognition site; sp, splotch; WS, Waardenburg syndrome; N-CAM, neural cell adhesion molecule; CAT, chloramphenicol acetyltransferase.

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In Pax-1, it has been suggested that Pax-3 could be a candidate target gene for Pax-3 regulation (2, 22). N-CAM and Pax-3 have overlapping expression patterns in the neural tube during mouse embryogenesis (1, 23). In addition, there appear to be significant differences in both the level of N-CAM expression and the types of N-CAM polypeptides present in the neuroepithelium of Sp/Sp homozygous mouse embryos as compared with wild-type embryos (22).

To test whether Pax-3 could affect the activity of the N-CAM promoter, we performed transient transfection experiments in NIH 3T3 fibroblasts. The reporter gene plasmid constructs contained various lengths of upstream sequences from the N-CAM gene (19, 20) fused to the bacterial CAT gene. In parallel with Pax-3, we also tested the effects of Pax-1, Pax-8, and Pax-6 in separate assays (Fig. 1 A and C).

**RESULTS**

**Inhibition of the N-CAM Promoter Basal Activity by Pax-3.** Recently, it has been suggested that Pax-3 could be a candidate target gene for Pax-3 regulation (2, 22). N-CAM and Pax-3 have overlapping expression patterns in the neural tube during mouse embryogenesis (1, 23). In addition, there appear to be significant differences in both the level of N-CAM expression and the types of N-CAM polypeptides present in the neuroepithelium of Sp/Sp homozygous mouse embryos as compared with wild-type embryos (22).

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![Fig. 1. Binding of Pax proteins to the N-CAM promoter and binding-independent transcription inhibition by Pax-3.](http://example.com/fig1.png)

**Figure 1.** Binding of Pax proteins to the N-CAM promoter and binding-independent transcription inhibition by Pax-3. (A) Schematic linear representation of Pax proteins used in this study. Numbers on the left refer to the number of amino acids N terminal to the corresponding PD. The PDs and HDs are boxed. Bars in these domains refer to the α-helices predicted by computer structure calculations. The circle between the PD and HD indicates the conserved octapeptide which is absent in Pax-6. (B) Structure of the N-CAM-CAT (N-CAM-Sty) reporter construct used for the transfection experiments shown in C. Numbers indicate nucleotide positions relative to the translation start site, and arrows indicate the transcription start sites as determined previously (19). The box (−140 to −12) indicates the DNA fragment which was used for the DNA binding experiment in D. (C) Three micrograms of the N-CAM-Sty reporter plasmid construct (shown in B) was cotransfected into NIH 3T3 cells with 3 μg of the indicated Pax expression vectors or with the expression vector without insert as a control (lane C). A quantitative evaluation of CAT activity is shown at the bottom. (D) Binding of the N-CAM promoter DNA fragment indicated by the boxed box in B to Pax proteins in a gel shift experiment using 300 ng of sperm DNA as a competitor for nonspecific DNA binding. The Pvu1-A variant of Pax-3 is described in Fig. 2A. The Pax-3/Sp variant corresponds to a deletion in the region between the PD and HD of Pax-3 (see ref. 15). The control extracts in lane C do not contain Pax proteins. F refers to the free DNA probe.

Analysis of the CAT activity from extracts of NIH 3T3 fibroblasts cotransfected with the reporter gene plasmid constructs and the expression vectors revealed a slight stimulation of N-CAM promoter activity by Pax-8 and Pax-6 (Fig. 1C). The basal level of transcription was not significantly affected upon Pax-1 cotransfection. However, N-CAM promoter activity was significantly reduced upon cotransfection of Pax-3. A typical example is shown in Fig. 1C with the N-CAM promoter fragment spanning from position −413 to +53 relative to the N-CAM translational start codon ATG (N-CAM-Sty in Fig. 1B). Similar results were obtained with various upstream deletions of the N-CAM promoter (data not shown).
To determine how the effect of Pax proteins on the N-CAM promoter is mediated, we dissected the promoter region (from −650 to +53) into short DNA fragments and assayed these for binding to Pax proteins in gel shift assays (Fig. 1D). A 128-bp DNA segment (nucleotide −140 to nucleotide −12) located upstream of the translation initiation ATG codon of the mouse N-CAM gene and downstream from the transcription start sites (see Fig. 1B, boxed area), was specifically bound by Pax-1, Pax-8, Pax-6, and the truncated Pax-3 protein, Pvu1-Δ, which contains the intact PD (Fig. 2A), but not by the wild-type Pax-3 or the Pax-3 splotch variant Pax-3/Sp (15). Thus, Pax-3 is able to inhibit N-CAM promoter activity, yet direct binding to the promoter region was not detected.

**Binding of Pax-3 to the PRS-1 Sequence.** To demonstrate the Pax-3 DNA-binding activity in the extracts that we used in the experiments with the N-CAM promoter, we performed gel mobility shift assays using the same extracts and the PRS-1 oligonucleotide as template. The PRS-1 sequence is derived from the Drosophila even skipped promoter and was shown to bind Pax-3 and other HD-containing proteins (15, 21). The PRSs are designated by numbers (21). Fig. 2C shows the sequences of PRS-1 and PRS-9, which both bind Pax-3 with similar affinities (15). In the following experiments we compared the binding of the wild-type Pax-3 with the binding properties of some Pax-3 truncated derivatives (Fig. 2A) in qualitative gel shift assays. As shown in Fig. 2B, the full-length Pax-3 protein binds to the PRS-1 sequence. The mutant Δ-Hinc, which lacks five amino acids at the N terminus of the PD, is still able to bind PRS-1, whereas further deletions into the PD abolish DNA binding. C-terminal deletions and the insertion of three amino acids within the conserved octapeptide (Pax3-Moct) do not affect the PRS-1 DNA binding. Interestingly, a Pax-3 mutant containing an insertion of 11 amino acids within the turn of the HD (Pax3-MHD) is still able to bind PRS-1 DNA. These results suggest that the PD of Pax-3 is essential for the sequence-specific binding of DNA. N-terminal deletions were sufficient to disrupt binding of Pax-3 to the PRS-1 sequence. No stable protein–DNA complexes could be detected even though these N-terminal deletions contained an intact HD and the recognition sequence contained an ATTAC motif.

**Pax-3 Concentration-Dependent Transcriptional Activation and Mapping of the Pax-3 Transcription-Modulating Domains.** Since the effect of Pax-3 on the transcription of the N-CAM promoter in cell culture experiments was not specific, we assayed specific transcriptional modulation by the Pax-3 protein by cotransfecting NIH 3T3 cells with a reporter gene plasmid construct carrying six copies of PRS-9 (see Fig. 2C for the sequence of PRS-9) attached to the TK-CAT gene, and increasing amounts of the Pax-3 expression vector (Fig. 3). PRS-9 (like PRS-1) can be specifically bound by Pax-3 (15) and thereby serve as a specific reporter to study transcriptional modulation by Pax-3. Cotransfection of 0.1 or 0.2 μg of the effector Pax-3 plasmid increased CAT transcription up to 8-fold. However, further increments (0.4–4.0 μg) of trans-

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**Fig. 2.** DNA-binding characteristics of wild-type and mutant Pax-3 proteins. (A) Schematic presentation of a linear Pax-3 structure on the top and the different mutants tested for DNA binding. (B) Whole-cell extracts from COS-7 cells (7 μg) expressing various Pax-3 protein segments (identified at the top of each lane and illustrated in A) were assayed for binding to a double-stranded PRS-1 oligonucleotide (C) in a gel shift assay using 300 ng of sperm DNA as a nonspecific competitor. F indicates the free template and C on the left side of the figure indicates the specific complexes between PRS-1 and the various Pax-3 derivatives. A faster-migrating complex is probably due to a specific degradation product of Pax-3 and comigrates with a nonspecific band. (C) Partial sequence of the PRS oligonucleotides used in this study. The ATTAC and GTTCC (PRS-1) or GATT (PRS-9) motifs are shown in boldface.

**Fig. 3.** Pax-3 activates transcription through multimerized PRS sequences. Six copies of the PRS-9 oligonucleotide (Fig. 2C) were linked to the thymidine kinase (TK) promoter (−105 to +57)/CAT gene. This reporter gene plasmid construct (3 μg) was cotransfected into NIH 3T3 cells along with different amounts of Pax-3 expression vector as indicated above the CAT assay. The expression vector without Pax-3 sequences was used to bring the total amount of the human cytomegalovirus promoter/enhancer-containing plasmid to 4 μg. Fold induction values over the basal CAT activity (control lane without Pax-3) are shown at the bottom.
fected effector plasmid decreased CAT activity. In contrast, the profile of transcriptional modulation by using a mutated PRS-reporter construct that cannot bind Pax-3 shows a gradual decrease in the CAT transcription with increasing amounts of cotransfected Pax-3 expression vector (data not shown). These data suggest that in the presence of functional PRS promoter elements, Pax-3 functions as a transcriptional activator over an unusually narrow concentration range, whereas Pax-3 concentrations above this narrow range inhibit transcription nonspecifically.

To map the domains of Pax-3 that are involved in transcription modulating activities, the full-length protein or various portions of the Pax-3 protein (Fig. 4A) were fused to the DNA-binding domain of the yeast GAL4 protein (GAL4 amino acids 1-147; ref. 18). For our reporter gene plasmid, we used 17M2-TK-CAT, which contains two copies of the GAL4-binding site linked to the TK-CAT gene (17).

Analysis of the CAT activity in NIH 3T3 fibroblasts cotransfected with various GAL4 fusion proteins and the reporter gene basal transcriptional activity in all constructs that included the N-terminal region of Pax-3 (Fig. 4B). The intact continuous segment of the first 90 amino acids of Pax-3 (GAP3-NK) displays a strong transcription-inhibiting activity. This region includes 57 amino acids of the PD and the first α-helix. A slight decrease in basal CAT activity was also detected when effector plasmids that express parts of (GAP3-CP1) or the complete HD (GAP3-CP2) were used. Whereas no transcriptional stimulation was evident with GAL4 chimeric proteins containing the PD or the HD, significant stimulation of basal CAT activity was observed with the GAP3-KB fusion protein, which contains the last 78 C-terminal amino acids of Pax-3. This indicates that the C terminus contains a transcription-activating domain when transferred to a heterologous protein.

**DISCUSSION**

**Specific DNA Binding by Pax-3.** Since in vivo target genes of Pax-3 are presently unknown, DNA-binding experiments have been performed by using PRS-1 as the standard DNA template. PRS-1 contains an ATTA motif recognized by the HD and a GTTCC motif bound by the PD (15). Efficient binding of Pax-3 requires the presence of both DNA-binding domains and both DNA recognition sequences. However, deletion of the HD produces a truncated PD (Pvu1-Δ) that can bind to the GTTCC site. These results suggest that the Pax-3 PD can bind to DNA independently of the HD. Independent DNA binding by the Pax-3 HD, although theoretically possible, has not been detected so far. This is possibly due to the lack of an appropriate DNA recognition sequence. The insertion of three amino acids within the conserved octapeptide sequence between the PD and the HD does not affect the binding of Pax-3 to PRS-1. Furthermore, an insertion of 11 amino acids in the turn between the second and third α-helix of the HD does not disrupt the binding of Pax-3 to PRS-1. Thus, it appears that a defect in the HD can be compensated by DNA binding of the PD through the GTTCC sequence. Such compensatory DNA binding activities in Pax-3 may also hold true for defects in the PD. For example, by analyzing the DNA-binding activity of the WS Brazil mutation, in which a highly conserved proline in the PD is exchanged for a leucine, we found that the PD alone, as a separate entity, cannot bind to the GTTCC sequence, whereas, in the context of the wild-type Pax-3 protein, it does bind (15). This indicates that the presence of the HD may compensate for the defect in the PD.

**Transcriptional Regulation by Pax-3.** The results presented here provide evidence that Pax-3 has the potential to modulate transcriptional regulation either by activating or by inhibiting promoter activity.

A domain responsible for a strong transcription inhibition is located in the first 90 N-terminal amino acids of Pax-3, which include the first 57 amino acids of the PD. This domain can function as a transcriptional inhibitor independent of other remaining portions of the Pax-3 protein, as it can be transferred onto a heterologous GAL4 DNA-binding domain. A lesser degree of transcription inhibition was also detected with forms of the truncated Pax-3 protein, including part of or the complete HD, when fused to the GAL4 DNA-binding domain. Interestingly, Pax-3, which is thus far the only member of the Pax family shown to have a transcription-inhibiting activity, has 33 N-terminal amino acids preceding the PD. We found that these 33 N-terminal amino acids are essential for Pax-3-mediated transcription inhibition. Pax-1 has only 2 additional N-terminal amino acids, Pax-8 has 8, and Pax-6 has 3 (Fig. 1A). Since the PD sequences are highly conserved among the Pax proteins, it will be of interest to test whether fusion of these 33 amino acids to other Pax proteins can confer transcription-inhibiting activity to them. Nevertheless, searching the protein data base with the Pax-3 transcription-inhibiting domain did not reveal any significant homology to other proteins except the PD homologs.
Pax-3 also inhibits the basal transcription of N-CAM reporter constructs in cellular cotransfection experiments. Since we observe no binding by the native Pax-3 protein in our studies, we cannot establish that N-CAM is a target gene of the wild-type Pax-3 protein. Thus, transcription inhibition by Pax-3 may be due to "squelching" through destabilization of the formation of preinitiation complexes, thereby interfering with the interactions between positively acting transcription factors and the general transcription machinery (24, 25). However, we have shown that the PvuI-Δ protein, which contains the intact PD of Pax-3 and is very similar to the sploch $Sp^{3H}$ Pax-3 protein product (2), can bind to the N-CAM promoter. Thus, since the PD of Pax-3 can bind to the N-CAM promoter, we cannot exclude the possibility that the wild-type Pax-3 can also bind to the N-CAM promoter under other (and perhaps even physiological) conditions, by stabilizing its DNA binding through interaction with other accessory proteins. Since Pax-1, Pax-6, and Pax-8 were shown to bind the N-CAM promoter, it is a compelling prospect that N-CAM is a downstream target of these Pax gene products.

We have also defined a transcription-activating domain in the Pax-3 protein that includes the 78 C-terminal amino acids. It is rich in proline and also in serine and threonine and it resembles the activating domains of Oct-2 (26) and CTF-1 (27). This transactivation domain can be transferred to a heterologous GAL4 DNA-binding domain and still function as an activator. Wild-type Pax-3 can also utilize this function to activate transcription at low concentration in a DNA-binding site-dependent manner.

Transcriptional-Modulating Activities of Pax-3 and the Semidominant Phenotype. The affected tissues in heterozygous sploch and WS1 patients are similar, and it has been suggested that part of the phenotype is caused by developmental defects in the neural crest. Homozygous WS has not been unambiguously demonstrated in humans and, by comparison to homozygous sploch, is likely to be lethal. Heterozygous sploch mice have pigmentation defects and, in some genetic backgrounds, facial appearances reminiscent of WS1. In general, WS1 patients (heterozygous) are much more severely affected than heterozygous sploch mice. Combining the phenotypic features of all Pax-3 $Sp$ and WS heterozygous mutants and considering that similar phenotypes result from molecular defects ranging from single point mutations to large deletions, we can exclude the possibility that the mutated allele participates in the phenotype by interfering with the function of the wild-type allele in heterozygotes, since large deletions produce comparable phenotypes. Considering both WS and $Sp$ mutants as being loss-of-function mutations, a very important question arising from the Pax-3 (PAX3) mutants is how to explain the partial phenotype seen in Pax-3 (PAX3) heterozygotes. Our results in Fig. 4 suggest that different concentrations of Pax-3 can specify its transcriptional effector function. Low amounts of Pax-3 expression plasmid lead to transcriptional activation, whereas high amounts result in inhibition. These different responses are elicited over a narrow concentration range, as a 2-fold increase in Pax-3 levels above the threshold level is sufficient to convert a stimulated form to a repressed one and vice versa. Thus, predetermined thresholds of Pax-3 may be involved in establishing cell fate in vivo, which may then be changed by slightly increasing or decreasing the level of Pax-3.

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