

# Altered regulation of platelet-derived growth factor receptor- $\alpha$ gene-transcription *in vitro* by spina bifida-associated mutant Pax1 proteins

(neural tube defects/embryonal carcinoma/osteosarcoma)

PAUL H. L. J. JOOSTEN\*<sup>†</sup>, FRANS A. HOL<sup>‡</sup>, SYLVIA E. C. VAN BEERSUM<sup>‡</sup>, HEIKO PETERS<sup>§</sup>, BEN C. J. HAMEL<sup>‡</sup>, GIJS B. AFINK<sup>¶</sup>, EVERARDUS J. J. VAN ZOELLEN\*, AND EDWIN C. M. MARIMAN<sup>‡</sup>

\*Department of Cell Biology, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands; <sup>§</sup>Institute Mammalian Genetics, GSF-National Research, Center for Environment and Health, 85764 Neuherberg, Germany; <sup>‡</sup>Department of Human Genetics, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands; and <sup>¶</sup>Department of Pathology, University Hospital, Uppsala University, S-75185 Uppsala, Sweden

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**ABSTRACT** Mouse models show that congenital neural tube defects (NTDs) can occur as a result of mutations in the platelet-derived growth factor receptor- $\alpha$  gene (*PDGFR $\alpha$* ). Mice heterozygous for the *PDGFR $\alpha$* -mutation *Patch*, and at the same time homozygous for the *undulated* mutation in the *Pax1* gene, exhibit a high incidence of lumbar spina bifida occulta, suggesting a functional relation between *PDGFR $\alpha$*  and Pax1. Using the human *PDGFR $\alpha$*  promoter linked to a luciferase reporter, we show in the present paper that Pax1 acts as a transcriptional activator of the *PDGFR $\alpha$*  gene in differentiated Tera-2 human embryonal carcinoma cells. Two mutant Pax1 proteins carrying either the *undulated*-mutation or the Gln  $\rightarrow$  His mutation previously identified by us in the *PAX1* gene of a patient with spina bifida, were not or less effective, respectively. Surprisingly, Pax1 mutant proteins appear to have opposing transcriptional activities in undifferentiated Tera-2 cells as well as in the U-2 OS osteosarcoma cell line. In these cells, the mutant Pax1 proteins enhance *PDGFR $\alpha$* -promoter activity whereas the wild-type protein does not. The apparent up-regulation of *PDGFR $\alpha$*  expression in these cells clearly demonstrates a gain-of-function phenomenon associated with mutations in Pax genes. The altered transcriptional activation properties correlate with altered protein–DNA interaction in band-shift assays. Our data provide additional evidence that mutations in Pax1 can act as a risk factor for NTDs and suggest that the *PDGFR $\alpha$*  gene is a direct target of Pax1. In addition, the results support the hypothesis that deregulated *PDGFR $\alpha$*  expression may be causally related to NTDs.

Neural tube defects (NTDs) form a major group of congenital malformations with an average incidence of  $\approx 1$  per 1,000 pregnancies. NTDs are generally accepted to represent multifactorial traits with genetic and environmental factors contributing to their aetiology. To date, very little is known about the identity of the genetic factors involved, although several candidate genes have emerged from studies of mutant mouse strains which display NTD or related malformations (1).

*Pax1* is a member of the Pax gene family of developmental control genes, which encode transcription factors that contain a DNA-binding “paired domain” (2). The gene is highly conserved between species and there is a 100% conservation between the paired domains of murine *Pax1* and its human counterpart *PAX1* at the amino acid level (3, 4). The mouse mutant *undulated* (*un*) represents a recessive missense muta-

tion in the paired domain of the *Pax1* gene, which results in a protein with altered DNA-binding affinity and transcriptional activity (5, 6). Analysis of the *un* phenotype has shown that Pax1 is essential for normal vertebral development (7, 8). Although these mice do not display spina bifida (*sb*), a high incidence of lumbar *sb* was observed in double mutants resulting from a cross between *un* and *Patch* (*Ph*) indicating that Pax1 can be involved in NTD aetiology (9). Moreover, we recently identified a missense mutation in the paired domain of the human *PAX1* gene in a patient with *sb*, suggesting that mutations in this gene may also play a role in human NTD (10).

The *PDGFR $\alpha$*  gene is located within a 50–400-kb region that has been deleted in the mouse mutant *Patch* (11). Heterozygotes are characterized by patches of white fur and have an intact axial skeleton, whereas homozygous embryos display occult *sb* involving the entire spinal column and die during early embryogenesis (12). Targeted inactivation of the murine *PDGFR $\alpha$*  gene results in mice with *sb* at the thoracic level (13). Recently, we have cloned and characterized the human *PDGFR $\alpha$*  promoter and shown that the 5′-flanking region together with the noncoding exon-1 acts as a functional promoter for the 6.4-kb full length receptor transcript (14, 15).

The observed NTD-phenotype of the double-mutant mice with the (*un/un, Ph/+*) genotype, suggests an interaction between Pax1 and *PDGFR $\alpha$* . This suggestion is supported by the observation that *PDGFR $\alpha$* -expression overlaps with Pax1 expression during embryonic development, particularly in the sclerotome, which forms the vertebral column (9). *PDGFR $\alpha$* -mutated mouse strains show no effect on Pax1-expression in the segmented paraxial mesoderm (13). Therefore, Pax1 may act as an upstream regulator of the *PDGFR $\alpha$* . Consequently, both *Pax1* and *PDGFR $\alpha$*  are considered to be candidate genes for *sb*.

Chalepakakis *et al.* (6) have shown Pax1 to bind to a specific DNA sequence: recognition sequence 4 (RS4). In the present study, we show that the *PAX1* mutation previously identified in a patient with *sb*, affects the RS4-binding properties of the protein. In addition, we demonstrate that wild-type (wt) and mutated Pax1 proteins have different effects on *PDGFR $\alpha$*  transcription in Tera-2 embryonal carcinoma cells in a differentiation-dependent manner and in the U-2 (OS) osteosarcoma cell line. The data obtained are discussed within the hypothesis that deregulated *PDGFR $\alpha$*  transcription may be causally related to NTD, including *sb*.

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: NTDs, neural tube defects; *PDGFR $\alpha$* , platelet-derived growth factor receptor  $\alpha$ ; EC, embryonal carcinoma; RA, retinoic acid; wt, wild type; *un*, *undulated*; *sb*, spina bifida; *Patch*, *Ph*.

<sup>†</sup>To whom reprint requests should be addressed. e-mail: P.Joosten@sci.kun.nl.

## MATERIALS AND METHODS

**Pax1 Constructs.** Expression constructs containing the full-length murine *Pax1* cDNA as well as the full-length *Pax1-undulated* construct (6) were kindly provided by R. Balling (Munich, Germany). To generate the sb-Pax1 mutant, the Gln at position 42 of the paired domain was replaced by a His corresponding to the mutation previously found in a patient with sb (9). Mutagenesis experiments were carried out by using the Altered Sites System kit (Promega) according to the manufacturer's protocol by using oligonucleotide primer 5'-GAGACCCGACGGTGCCTACTGAT-3'. Presence of the mutation (bold) was confirmed by dyedeoxy termination cycle sequencing (ABI) of the constructs on an ABI370A automated sequencer.

**In Vitro Transcription/Translation.** The synthesis of wt Pax1, sb-Pax1, and un-Pax1 proteins was performed by using the TnT-T7-Coupled Promega Rabbit Reticulocyte Lysate System (Promega) according to the manufacturer's protocol. For this purpose, a T7-promoter was introduced by PCR on Pax1 expression constructs, which encode Pax1 (wt-Pax1), un-Pax1, or sb-derived Pax1 (sb-Pax1), respectively. Forward primer (including T7 promoter and start codon) 5'-CGCTA-ATACGACTCACTATAGGAACAGACCACCATGGAGC-AGACGTACCGAAGTGAAC-3' and reverse primer 5'-G-GCTGTGGCTCTGTGAGAG-3' (located in 3' untranslated region; ref. 6) were used to generate the wt-Pax1-, un-Pax1-, and sb-Pax1-encoding templates. These templates were used subsequently for *in vitro* transcription/translation.

**Generation of Pax1-Specific Antibodies.** Pax1-specific polyclonal antibodies were obtained by immunizing two rabbits with a Pax1-specific peptide coupled to maleimide-activated keyhole-limpet-hemocyanin (Pierce) according to the manufacturer's instructions and established protocols (16). The Pax1-specific peptide (NH<sub>2</sub>-FKHREGTDRKPPSPG-(COOH) corresponds to amino acid number 327–341 of the Pax1-protein (6). A total of 1 mg of coupled protein was mixed with incomplete Freund's adjuvans and used for initial immunization. Booster injections with 100 µg of coupled protein were given in 4-week intervals. Total serum was collected 11 days after the fourth boost, designated as 289-IV, and used in these studies.

**Western Blotting.** Protein extracts were separated on a 10% SDS-polyacrylamide gel and subsequently transferred to a nitro-cellulose membrane, which then was pretreated for 20 min with blocking buffer (0.4% gelatine/3% BSA/10 mM Tris, pH 7.5/350 mM NaCl). The membrane is incubated subsequently with the Pax1 antibody (diluted 1:200 in RIA buffer: 10 mM Tris, pH 7.5/160 mM NaCl/1% Triton/0.1% SDS/0.5% sodiumdesoxycholate) for 1 hr at room temperature and treated with goat-anti-rabbit alkaline phosphatase conjugate for 1 hr at room temperature (Bio-Rad; dilution 1:5,000 in RIA buffer). The membrane was then treated with APB buffer (100 mM Tris pH 9.5; 100 mM NaCl; 5 mM MgCl<sub>2</sub>) and subsequently stained by using bromochloroindolylphosphate and nitrobluetetrazolin as substrates.

**Electrophoretic Mobility Shift Assay.** In these assays, a <sup>32</sup>P-3' end-labeled PRS4 oligonucleotide was used as described (6) (5'-TGGGCTCACCGTTCGCTCTAGATATCTCGA-3'). The assays were performed essentially as described (17, 18).

**Cell Culture.** Tera-2 embryonal carcinoma (EC) cells (Clone 13) were grown in  $\alpha$  modification of minimal essential medium lacking nucleosides and deoxynucleosides, supplemented with 10% (vol/vol) fetal calf serum and 44 mM NaHCO<sub>3</sub> in a 7.5% CO<sub>2</sub> atmosphere at 37°C. Undifferentiated Tera-2 EC cells were seeded at high density ( $5.0 \times 10^4/\text{cm}^2$ ), and differentiation of cells was induced by the addition of retinoic acid (RA) (5 µM) 12 h after the cells were seeded at low density ( $5.0 \times 10^3/\text{cm}^2$ ), and maintained in this medium

for 7 days before further use. U-2 OS cells were seeded at high density ( $5.0 \times 10^4/\text{cm}^2$ ) and grown in DMEM/nutrient mix F12 (1:1) with supplements and conditions as mentioned above.

**Transfection, Luciferase, and  $\beta$ -Galactosidase Assays.** The -944/+118 human PDGFR $\alpha$ -promoter construct (14, 15) and expression vectors containing no insert, wt-Pax1-, sb-Pax1-, and un-Pax1-encoding inserts, respectively, were transiently transfected into Tera-2 cells or U-2 OS cells by using the calcium phosphate coprecipitation method (19). Luciferase activity was detected 48 h after transfection (Luciferase assay kit, Promega). The luciferase activity was corrected for transfection efficiency by measuring the  $\beta$ -galactosidase activity due to a cotransfected cytomegalovirus promoter-driven *lacZ* gene construct. Transfections were done in duplicate with different batches of DNA.

## RESULTS

**Production of wt and Mutant Pax1.** To test the functionality of the different Pax1 proteins, full-length murine Pax1 proteins were generated in an *in vitro* transcription/translation system. In addition to the wt-Pax1 protein (wt-Pax1), two NTD-associated mutant proteins were synthesized also. The first one (sb-Pax1) contained the Gln  $\rightarrow$  His substitution that was previously identified by us at position 42 of the paired domain in a patient with sb (10). The second mutant protein (un-Pax1) contains the Gly  $\rightarrow$  Ser replacement at position 15 of the paired domain that is known to be responsible for the *un* phenotype in mice (5).

To show that full-length proteins are synthesized, we performed a Western blot analysis. For this purpose polyclonal antibodies were generated recognizing the C-terminal part of Pax1, which is not affected in the mutated proteins. These antibodies detect a protein of the predicted size of 40 kDa in protein extracts of the developing vertebral column of 13.5-day-old mouse embryos (Fig. 1). In contrast, no Pax1 protein was detectable in the extract of the *Un<sup>s</sup>* mouse embryo in which the *Pax1* gene is deleted (7), showing that the antibodies are Pax1-specific. By using these antibodies, the three *in vitro*-generated Pax1 proteins are detectable at the same level as the wt-Pax1 protein (Fig. 1), thereby demonstrating that full-length proteins have been synthesized.

**Binding Properties of wt and Mutant Pax1.** To study the DNA-binding ability of the different Pax proteins, electrophoretic mobility shift assays were carried out by using the PRS4 consensus-binding sequence for Pax1 (6). Fig. 2 shows that with wt-Pax1 the formation of a specific DNA-protein complex causes a clearly detectable band-shift. Using the antibody, Pax1 was identified within this complex (not shown). In contrast, no bandshift was detected with un-Pax1, which is in accordance with the earlier work of Chalepakos *et al.* (6). In this context one should realize, however, that un-Pax1 was shown to be able to bind to other sequences. Remarkably, sb-Pax1 induces a shift but with a different mobility from that induced by wt-Pax1. Because both proteins have an identical molecular size (Fig. 1), sb-Pax1 apparently binds to the DNA-sequence in a slightly different conformation compared with wt-Pax1. These altered binding characteristics may have consequences for the proper functioning of the Pax1 transcription factor.

**Effect of wt and Mutant Pax1 on PDGFR $\alpha$  Transcription.** As already mentioned, the phenotype of (*un/un*, *Ph/+*) double mutants suggests that Pax1 and PDGFR $\alpha$  participate in the same functional cascade. One possibility is that Pax1 acts as an upstream regulator of PDGFR $\alpha$ . To assess the effect of the various Pax1 proteins on the transcriptional activity of the PDGFR $\alpha$  promoter, plasmids encoding these proteins were cotransfected with the -944/+118 human

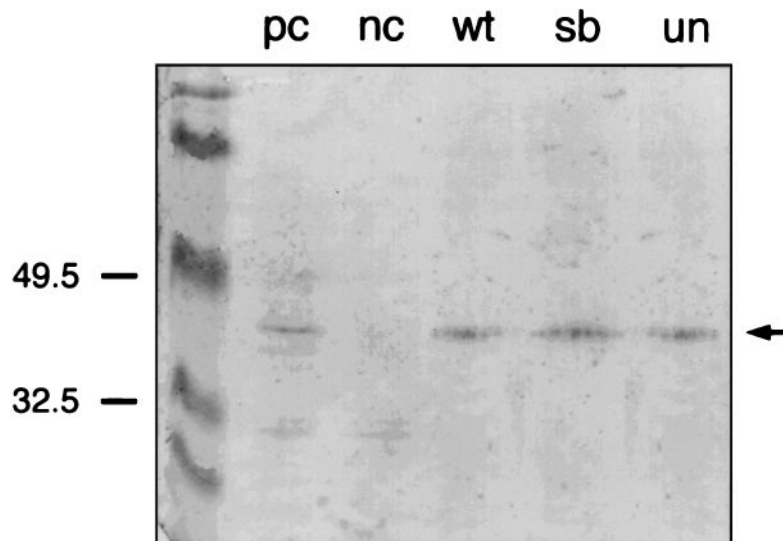


FIG. 1. *In vitro* transcription and translation of Pax1 and derived mutants. The *in vitro* transcription/translation products of wt-Pax1 (wt), sb-derived-Pax1 (sb), and un-Pax1 (un) were detected on Western blot by a specific anti-Pax1 antibody. As a positive (pc) and negative control (nc) total protein extract from the developing vertebral column of 13.5-day-old wt and a homozygous *undulated shorttail* (*Un<sup>S</sup>*) embryos were used, respectively. In *Un<sup>S</sup>* mutant mice, the Pax1 gene has been deleted completely. The antibody detects a predicted band of  $\approx 40$  kDa, which is not present in the negative control. No Pax1 was detected in the transcription/translation lysate (not shown).

*PDGFR $\alpha$*  promoter linked to the luciferase gene. Transfection studies were carried out in Tera-2 cells. In their undifferentiated state, these EC cells resemble cells in early stages of human development. Only after *in vitro* differentiation by RA these cells express a functional *PDGFR $\alpha$*  (20). No endogenous expression of Pax1 was observed in both Tera-2 EC and Tera-2 RA cells by reverse transcription-PCR analysis (not shown). Because Pax1 is one of the earliest markers for sclerotome differentiation, additional transfection experiments were carried out in the U-2 OS osteosarcoma cell line. These cells have endogenous *PDGFR $\alpha$* -expression (21). Fig. 3A shows that wt-Pax1 induces up to threefold stimulation of promoter activity in Tera-2 RA cells. Un-Pax1 is ineffective in increasing promoter activity in these cells, whereas sb-Pax1 has intermediate activity. These results show that an intact Pax1 protein is essential to

efficiently promote *PDGFR $\alpha$*  transcription in Tera-2 RA cells. Interestingly, in Tera-2 EC cells a significant increase in activity of the *PDGFR $\alpha$*  promoter was observed with sb-Pax1 whereas only little effect was seen with un-Pax1 (Fig. 3B). Fig. 3C shows that in U-2 OS sb-Pax1 and un-Pax1 both induce promoter activity, the latter being the most potent activator under these conditions, whereas wt-Pax is again ineffective. These results show that wt and mutated Pax1 proteins can have opposing transcriptional activities, depending on the specific transcriptional background in different cell types. Moreover, it shows that mutations in Pax1 can result in a gain-of-function with respect to transcriptional activity of the protein.

**DISCUSSION**

In the present study, we have shown that the Gln  $\rightarrow$  His mutation in PAX1, previously identified by us in a patient with sb, is associated with altered *in vitro* DNA-binding properties of the full length protein. Our results suggest that this mutation affects the functionality of the PAX-1 protein and, therefore, may have contributed to the phenotype observed in the patient.

In addition, we investigated the influence of Pax1 on *PDGFR $\alpha$*  transcription. We showed that wt-Pax1 protein enhances *PDGFR $\alpha$*  transcription in differentiated Tera-2 human embryonal carcinoma cells (Fig. 3A), whereas two mutant Pax1 proteins carrying either the *undulated* mutation (un-Pax1) or the Gln  $\rightarrow$  His mutation (sb-Pax1), were not or less effective, respectively. Surprisingly, Pax1 mutant proteins appear to have opposing transcriptional activities, depending on the cell differentiation status in the Tera-2 cells, as well as in the U-2 OS osteosarcoma cell line. (Fig. 3 B and C). These activities of the mutated Pax-1 proteins are the first examples of mutations in a Pax gene that can be associated with a gain-of-function phenomenon. This could have yet unknown consequences during embryonic development and may play a role in the pathogenic mechanism underlying specific types of NTD.

The above observations support the hypothesis that Pax1 is an upstream regulator of *PDGFR $\alpha$*  and that deregulated *PDGFR $\alpha$*  gene transcription may be causally related to sb. In

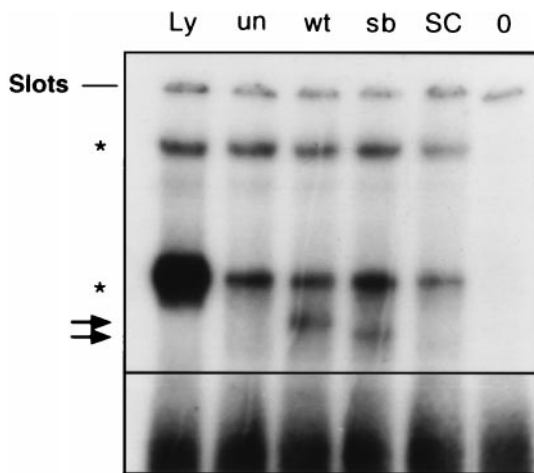


FIG. 2. Electrophoretic mobility shift assays with Pax1 and derived mutants. *In vitro* transcription/translation products of wt-Pax1, sb-derived Pax1, and un-Pax1 were assayed for binding activity on the PRS4 probe derived from the e5 site in the *Drosophila* even-skipped promoter. The lanes Ly, un, wt, sb, SC, and 0, correspond to incubation of the probe with reticulocyte lysate, un-Pax1, wt-Pax1, sb-Pax1, and wt-Pax1 + unlabeled PRS4 probe in a 500-fold excess, and H<sub>2</sub>O, respectively. The Pax1 bands are indicated with arrows. Nonspecific bands are indicated with asterisks.

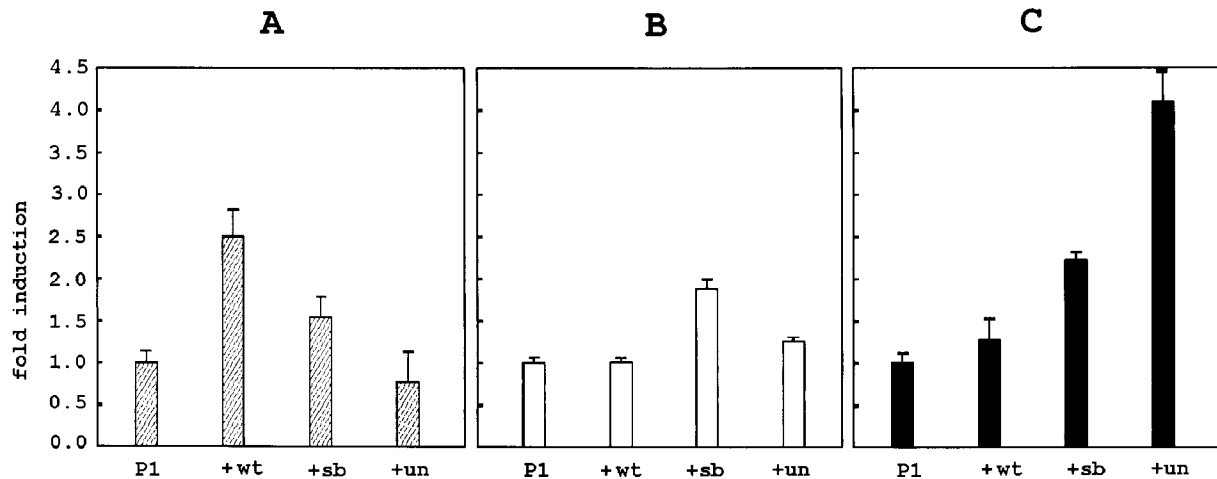


FIG. 3. Regulation of transcriptional activity of the PDGFR $\alpha$  promoter by Pax-1 and derived mutants. The activity of the  $-944/+118$  promoter luciferase construct (P1) is regulated differently by wt-Pax1, sb-Pax1, and un-Pax1 in RA-differentiated Tera-2 RA cells (A, hatched bars), undifferentiated Tera-2 EC cells (B, open bars), and U-2 OS (C, black bars). Values are presented as mean promoter activity relative to the activity of the  $-944/+118$  clone cotransfected with the expression construct without a Pax1 insert, which was set to 1. Error bars indicate the sample SD after four luciferase/ $\beta$ -galactosidase measurements.

this way, it can be explained that sb is not only observed in the targeted PDGFR $\alpha$  null mutation (13) and in homozygous *Patch* (*Ph/Ph*) mice (12) but also in *Patch* (*Ph/+*) heterozygotes with an additional Pax1 mutation (*un/un*) (9). Accordingly, the sb phenotype in the patient carrying the sb-Pax1 mutation may be the result of impaired regulation of PDGFR $\alpha$  transcription. In this context, the gain-of-function phenomenon could be very important for effectively deregulating PDGFR $\alpha$ -expression and thus could contribute as well to the sb phenotype. It is well realized, however, that NTDs including sb are a multifactorial trait and that other genes and/or environmental influences are likely to be involved.

We have previously shown that the human PDGFR $\alpha$  gene is able to give rise to multiple mRNA products as a consequence of alternative promoter use and alternative splicing (18, 22). In Tera-2 EC cells, a 1.5-kb and a 5.0-kb transcript have been identified, which are transcribed from a promoter in intron 12 of the gene (18). In contrast, in Tera-2 RA cells a 6.4-kb and a 3.0-kb transcript are present, for which transcription is driven by the promoter upstream of exon 1. Because the 6.4-kb transcript generates the functional, full length  $\alpha$ -receptor, this promoter has been used for the current activation studies with Pax1. The  $-994/+118$  region of the human PDGFR $\alpha$  promoter contains the larger part of the information required for tissue-specific regulation in transgenic mice, as previously shown by us (15). This promoter fragment contains multiple putative Pax1-binding site core elements (5'-GTTCC-3'; ref. 6).

Additional studies will have to indicate whether Pax1 actually binds to the PDGFR $\alpha$  promoter directly, or rather mediates its effect via interaction with other components of the transcription machinery. In the case of direct binding of the proteins to the PDGFR $\alpha$  promoter, the different activities could be explained by an altered cofactor dependency of the mutated proteins. In the case of an indirect influence of Pax1 proteins on PDGFR $\alpha$ -promoter activity, at least for undulated, it also might be possible that other genes are activated because of its altered DNA-binding specificity (6).

In summary, the present results emphasize the relevance of PDGFR $\alpha$  expression regulation during vertebral column development and support the model that impaired expression of this gene may be causally related to sb. Evidence from the transfection studies suggests that Pax1 is indeed an upstream regulator of PDGFR $\alpha$ , and mutations in Pax1 may result in a

gain-of-function of the protein. The different activities of the wt and mutant Pax1 proteins correlate with an altered conformation of the corresponding protein-DNA complex in the bandshift assays. The present data corroborate our earlier hypothesis that mutations in *PAX1* can act as risk factors in NTD but also present the PDGFR $\alpha$  gene as the central gene involved. Additional studies of this gene have to be performed to investigate whether mutations in PDGFR $\alpha$  contribute to human NTD, particularly to sb.

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