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Identification of a New Binding Motif for the Paired Domain of Pax-3 and Unusual Characteristics of Spacing of Bipartite Recognition Elements on Binding and Transcription Activation*

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Pax-3, a transcription factor that is required for development of the embryonic neural tube, neural crest, and somitic derivatives, contains two DNA-binding domains, a paired domain, and a paired-type homeodomain. Although Pax-3 binds to sequences related to the e5 element of the *Drosophila even-skipped* gene, the sequence requirements of an optimal Pax-3 response element have not been well characterized. Using both DNA-binding domains and a pool of random oligonucleotides, we identified a new paired box consensus motif, "GTTAT," which was located 1, 4, 5, 8, or 13 base pairs downstream of the homeobox binding motif, "ATTA." Binding analysis of these sequences demonstrated that the distance between recognition elements for the homeodomain and the paired domain affects affinity. Specifically, spacing elements 1 or 13 base pairs apart from each other conferred low affinity Pax-3 binding, whereas intermediate spacing (5 or 8 base pairs) conferred high affinity binding. Contrary to previous reports, oligonucleotides deleted for either the ATTA or the GTTAT could also be bound by Pax-3, although both sites were necessary for maximal affinity. Finally, transient transfections demonstrated that Pax-3 trans-activation correlated with binding affinity. Because the Pax-3-responsive genes identified to date contain almost exclusively low affinity binding sequences, our analysis indicates that they may be responsive to Pax-3 only when cellular levels are high.

Pax-3 encodes a bipartite DNA-binding transcription factor that is essential for vertebrate embryogenesis. The two DNA-binding domains consist of a 128-amino acid paired domain, which itself is comprised of two helix-turn-helix subdomains that bind to distinct DNA recognition sequences (1, 2), and a 61-amino acid paired-type homeodomain. In the mouse embryo, expression of *Pax-3* begins on day 8.5 in the dorsal neuroepithelium along the anterior-posterior axis (3). By day 9.5, *Pax-3* expression extends into the lateral dermomyotome, somitic mesoderm, and neural crest (3). The *Pax-3* gene is essential for differentiation of the neural tube and other *Pax-3*-expressing tissues, as mutation or deletion of the gene is responsible for the *Splotch* phenotype in mice (4–6), which is associated with exencephaly and/or spina bifida with 100% penetrance in homozygous embryos (7). Similarly, reduced expression of *Pax-3* in embryos of diabetic mice causes exencephaly (8), indicating that a threshold level of functional protein is critical for proper neural tube development. The amino acid sequence of the murine Pax-3 paired domain is 100% identical to the human PAX-3 paired domain, suggesting that it performs similar regulatory functions during embryogenesis of mice and humans (9). Indeed, mutations in the human *PAX-3* gene have been found in patients with Waardenburg Syndrome (9–11), an autosomal dominant phenotype characterized by neural crest defects, including pigmentation disturbances, lateral displacement of the inner canthus of the eye, and occasional deafness and mental retardation. Additional evidence of Pax-3's role in regulatory processes is that in humans, the pediatric solid tumor, alveolar rhabdomyosarcoma, appears to result from a genetic translocation which causes production of a fusion protein comprised of the DNA-binding domains of PAX-3 and the trans-activation domain of the FKHR protein (12–14).

The prototypical paired domain was first identified in the protein products of several *Drosophila* segmentation genes, including *paired* (*prd*) and *gooseberry* (*gsb*) (15–17). The *Drosophila* Prd protein binds specifically to the e5 sequence found in the promoter of the *Drosophila even-skipped* gene (18, 19). Like the *Drosophila* Prd protein, murine Pax-3 can bind specifically to the e5 sequence (3). Nuclease protection analysis has suggested that the Pax-3 homeodomain binds to an ATTA motif within the e5 sequence, and the paired domain binds to a GTTCC core motif (20), which is 5 bp[1] downstream of the ATTA. Study of the effects of base substitution within the paired domain binding element of e5 have indicated that Pax-3 binding can tolerate some sequence variation at the last three positions within the 5-bp core paired recognition element (9, 17). Although each of the DNA-binding domains of *Drosophila* Prd can bind to e5 recognition elements independent of the other (18, 21), albeit with lower affinity than binding by both domains together (2), previous studies have demonstrated that Pax-3 binding to e5 oligonucleotides requires both the homeodomain binding sequence and the paired domain binding sequence (20). Furthermore, localization of the paired domain and homeodomain recognition elements within 2–10 bp of each other has been found to be essential for Pax-3 binding (22). These results would indicate that a sequence, ATTA-N_{2-10}-

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[1] The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; GST, glutathione *S*-transferase; hGH, human growth hormone; nt, nucleotide(s); CMV, cytomegalovirus.

GTT/CAT/C is important for Pax-3 binding and transcription regulation. However, among the few genes that are positively or negatively regulated by Pax-3 in vertebrate embryos, including myelin basic protein, N-CAM, c-Met, and *myoD* (23–27), only the chicken *myoD* promoter contains an element with both the homeodomain and paired domain recognition sequences within the vicinity of one another that is thought to be required for binding. In the case of the N-CAM promoter, a Pax-3 variant containing just the paired domain is able to bind to a Pax-3-responsive region, but the intact protein is not (24). This suggests that additional analysis of the sequence determinants for Pax-3 binding must be performed to identify putative Pax-3 response elements in its downstream regulated genes.

We report here the identification of a new consensus sequence for Pax-3 binding from a pool of random oligonucleotides using both the DNA-binding domains of Pax-3. Oligonucleotides containing variations of the consensus sequence were evaluated for Pax-3 binding affinity and for trans-activation by Pax-3. From these results, a model in which the magnitude of transcriptional responsiveness of target genes depends on binding affinity is presented.

EXPERIMENTAL PROCEDURES

GST/Pax-3 Fusion Protein Construction with the Pax-3 DNA-binding Domains—The RF1-Pax-3 expression plasmid (28), kindly provided by George Chalepakis (Max-Planck Institute for Biophysical Chemistry, Germany), was used as a template for PCR amplification of a 775-bp region surrounding the complete paired box and paired-type homeobox of the Pax-3 cDNA using the forward and reverse primers, ACTGCGAATTCGCTGGAAGTG and TGACGGAATTCATCAGTTGATTGGC, respectively. Each primer was designed to contain an *Eco*RI restriction site at the 5′ terminus of the PCR product. PCR reactions contained 0.2 μM each of the above Pax-3 primers, 180 ng RF1/Pax-3 DNA, 0.5 mM dNTP, 1 × PCR buffer, and 1 unit of AmpliTaq (Perkin-Elmer). 30 cycles were performed using the following conditions: 95 °C for 2 min, 50 °C for 1 min, 72 °C for 1 min, followed by a 5 min extension at 72 °C. The entire PCR reaction was digested with *Eco*RI and electrophoresed on a 1.2% agarose gel, from which the 795-bp fragment was isolated using the Geneclean kit (Bio-101, Inc.) using the manufacturer's suggestions. This fragment was ligated into *Eco*RI-digested, dephosphorylated pGEX-3X plasmid (Amersham Pharmacia Biotech) and used to transform DH5α strain of *Escherichia coli* cells that had been made competent as described (29). The sequence of the resulting GST/Pax-3 fusion cDNA was confirmed by DNA sequence analysis (30) using a Sequenase version 2.0 kit (U. S. Biochemical Corp.).

GST/Pax-3 Production and Isolation—The GST/Pax-3 fusion protein was prepared using the GST gene fusion system (Amersham Pharmacia Biotech). Fusion protein production was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (Sigma) for 2 h, and protein was isolated and purified according to the manufacturer's suggestions. Samples from the initial bacterial cell lysate and the final bead eluates were analyzed by SDS-polyacrylamide gel electrophoresis (31) using a 10% polyacrylamide gel. High molecular weight prestained protein standards (Life Technologies, Inc.) were used to the estimate the sizes of the protein products. Following electrophoresis, protein gels were either fixed and stained with Coomassie Blue to visualize protein components (29) or used for Western blotting as follows. Protein gels were electroblotted with a Semi-Phor TE70 semi-dry blotter (Hoefer Scientific) onto Duralon UV nylon membranes (Stratagene). Membranes and filter paper were presoaked in transfer buffer containing 25 mM Tris, 200 mM glycine, 20% methanol, and 0.01% SDS, then electroblotted for 1 h at 200 mA. Membranes were rinsed in TBS (10 mM Tris, pH 8.0, 150 mM NaCl), then blocked for 1 h at room temp with rocking, in 1% dry milk in TBS. Membranes were incubated with 10 ml of a 1:100 dilution of mouse anti-GST antibody, kindly provided by Dr. Bill Kaelin (Dana Farber Cancer Institute), diluted in 1% dry milk/TBS solution, and rocked overnight at 4 °C. Following three subsequent 10 min washes with TBS, membranes were incubated with 20 ml of a 1:5000 dilution (100 milliunits) of goat anti-mouse IgG antibody solution (Oncogene Science) and rocked for 1 h at room temperature. Three additional 10-min washes with TBS were followed by a 1-min rinse with 1 × AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$). 30 ml of AP buffer were mixed with 49.5 μl of 5-bromo-4-chloro-3-indolyl phosphate, then 99 μl of 4-nitroblue tetrazolium chloride (both from Boehringer Mannheim), after which the solution was immediately added to the blot and rocked at room temp until color reaction appeared (approximately 2–3 min). Reactions were stopped by rinsing the blot with distilled H_2O, and blots were subsequently stored in aluminum foil to preserve color.

Assay for GST/Pax-3 DNA Binding Specificity—Ten nmol of double-stranded e5 oligonucleotide were prepared by annealing equal molar amounts of complementary oligonucleotides (GATCCTCGACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCCCTGCA and GGGAGCGGAACGGTGCTAATCGTGCGGTGCTGAGAGTCGAG) (3) in 100 μl containing 50 mM NaCl, 100 mM $NaPO_4$. Reactions were heated to 85 °C for 3 min, then the solution was allowed to cool slowly to room temperature. Approximately 50 pmol were used for 3′ end labeling reactions (29) with 5 units of Klenow fragment of DNA polymerase, using 2 μl of [^{32}P]dGTP (3000 Ci/mmol). DNA was phenol-chloroform- and chloroform-extracted, ethanol-precipitated, and resuspended in 10 μl of H_2O. Approximately 20 fmol of probe were incubated with 1 μl of eluted GST or GST/Pax-3 protein and 1 μg of poly(dI-dC) (Boehringer Mannheim), in 90 mM KCl, 0.4 mM dithiothreitol, 2 mg/ml bovine serum albumin, and 6.5% glycerol (32). Binding reactions were performed in the presence of unlabeled competitor DNA (e5, or E2, described below) at a 0-, 50-, 100-, or 200-fold molar excess and were carried out for 20 min at 30 °C and were electrophoresed for 2 h at 150 volts on a 0.75-mm 4% nondenaturing polyacrylamide gel in 0.25 × TBE. Gels were dried onto 3MM filter paper (Whatman) and exposed to film at −80 °C for 16–72 h.

Random Oligonucleotide Selection by Electrophoretic Mobility Shift Assay—The sequence of the random oligonucleotide was designed according to previously described flanking anchor sequences (33), for cloning. This sequence, CGGGCTGAGATCAGTCT*AGATCT*(N)$_{35}$GGATCCGAGACTGAGCGTCGTC, where the *Xba*I and *Bam*HI restriction sites are underlined, and the internal *Bgl*II restriction site is indicated in italics. An oligonucleotide containing the e5 sequence, and the flanking anchor sequences in the random oligonucleotide, was designed to be used as a control. The sequence of this oligonucleotide was: CGGGCTGAGATCAGTCT*AGATCT*GATCCTCGACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCCCTGCAGGATCCGAGACTGAGCGTCGTC, with internally designed restriction endonuclease digestion sites indicated as above. Double-stranded radiolabeled probes were synthesized by annealing each oligonucleotide with the following 3′ anchor primer: GACGACGCTCAGTCTCGGATCC, followed by extension with Klenow, as described above, in the presence of 1 μl of [^{32}P]dCTP in place of unlabeled dCTP. Probes were electrophoresed on an 8% nondenaturing acrylamide gel, and bands were excised and eluted overnight at 37 °C in 400 μl of acrylamide extraction buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS, and 10 mM Tris-HCl, pH 7.5) (34). DNA was phenol-chloroform- and chloroform extracted and precipitated with 0.25 M NaCl, 10 μg glycogen, and 3 × volume of 100% ethanol for 1 h at −80 °C. DNA was resuspended in 100 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and incorporated radioactivity was measured in counts/min (cpm) in a scintillation counter.

Binding site selection procedure was performed as described previously (35), with the following modifications. Between 12,000 and 15,000 cpm of probe were incubated with 1 μl of eluted GST/Pax-3 protein in a 20 μl of binding reaction containing 1 μg of poly(dI-dC), and 10 μl of GST fusion protein binding buffer (36) (50 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 10 μM $ZnSO_4$, mm dithiothreitol, and 50% glycerol), for 30 min, at 30 °C. Reactions were electrophoresed as described above. The initial binding reaction was electrophoresed on a nondenaturing polyacrylamide gel alongside a complex of GST/Pax-3 and radiolabeled e5 probe that was used as a migration control. The lane containing random oligonucleotide was excised from the gel at the same position as a GST/Pax-3·e5 complex, and bound DNA at that position was eluted from the gel, phenol-extracted, and precipitated as described above. Half of the eluted oligonucleotides were PCR-amplified in 50 μl of PCR reactions containing 0.2 μM each of a 5′ anchor primer, CGGGCTGAGATCAGTCTAGATCT, and the 3′ anchor primer used for second strand synthesis, 200 μM of each dATP, dTTP, and dGTP, 2 μM dCTP, 1 μl of [^{32}P]dCTP (3000 Ci/mmol), 2.5 mM $MgCl_2$, 1 × AmpliTaq buffer (Perkin-Elmer), and 5 units of AmpliTaq polymerase. 30 cycles of PCR were performed using the following cycling conditions: 94 °C, 30 s; 55 °C, 2 min; 72 °C, 30 s; followed by a 5-min extension at 72 °C. PCR reactions were electrophoresed on 8% nondenaturing polyacrylamide gels and exposed to x-ray film for 1–3 min. Probes were excised, eluted, precipitated, and counted, as described above. Approximately 12,000–15,000 cpm were used for a subsequent round of binding and amplification. This selection procedure was repeated for a total of four complete rounds of enrichment, and following the fourth round of selection, the final PCR products were digested with *Xba*I and *Bam*HI, purified on

an 8% polyacrylamide gel, and cloned into the *Xba*I-*Bam*HI sites of the pBluescript SK+ vector (Stratagene). The selected oligonucleotides are referred to as SAAB (selected and amplified binding sites) (35) sequences. Individual clones were selected and digested with *Bgl*II to verify the presence of insert. Sequencing of selected clones was performed by the dideoxy chain termination method (30) with the Sequenase version 2.0 kit (U. S. Biochemical Corp.), using the supplied M13 −40 reverse primer.

Competition Gel Mobility Shifts—Individual probes for gel shift assays were radiolabeled by PCR amplification using [^{32}P]dCTP, as described above for random oligonucleotide selection. DNA templates for PCR included approximately 10–100 pmol of double-stranded oligonucleotide (for the GST/Pax-3-selected random oligonucleotide pool, e5, e5seq, and e5spac) or 100 ng plasmid DNA from cloned saab oligonucleotides (for saab-1, saab-8, and saab-13). Probes were gel purified, as described above, and resuspended in 100 μl of TE. Specific activity was determined by counting 1 μl of resuspended probe in a scintillation counter and calculating molar amount of probe from percent incorporation of labeled dCTP. Five to ten fmol of ^{32}P-labeled probe were incubated with GST/Pax-3 protein as described above, in the presence or absence of unlabeled competitor oligonucleotide, as indicated in figure legends. Sequences for oligonucleotides used for probes in competition assays included e5, CGGGCTGAGATCAGTCTAGATCTATTAGCACCGTTCCGGATCCGAGACTGAGCGTCGTC; e5seq, CGGGCTGAGATCAGTCTAGATCTATTAGCACCGTTATGGATCCGAGACTGAGCGTCGTC; and e5 spac, CGGGCTGAGATCAGTCTAGATCTATTAAGTTCCGGATCCGAGACTGAGCGTCGTC. In addition, oligonucleotides were designed to the saab-8 sequence, base-substituted for either the ATTA or GTTAT motif. These two single site oligonucleotides were designated PDbs (containing the putative paired domain binding site): GACGACGCTCAGTCTCGGATCCTAATAATGAACATGTATGCCGTATATTTA**GTTAT**TAGATCTAGACTGATCTCAGCCCG and HDbs (containing the putative homeodomain binding site): CGGGCTGAGATCAGTCTAGATCTACGCCGTAAATATATAATATACATGTTCATT**ATTA**GGATCCGAGACTGAGCGTCGTC. Retained putative binding sites are shown in boldface, and mutated bases are underlined. Sequences of the homologous competitors for each selected oligonucleotide were AGCTTTAGTGAATTATGTTATTAAAATGGTCTAGAC (for saab-1), AGCTTTGTATATTATATATTTAGTTATGGTCTAGAC (for saab-8), and AGCTTATTATGTTTTACTAGATGTTATGGTCTAGAC (for saab-13). Reactions were analyzed on a 4% nondenaturing polyacrylamide gel, which was subsequently dried onto Whatman 3MM paper, and exposed to x-ray film at −80 °C, overnight.

Electrophoretic Mobility Shift Assay Quantitation—Dried gels from gel mobility shift assays were exposed on a phosphor screen for 16–72 h, and images were scanned on a PhosphorImager (Molecular Dynamics). Bands were analyzed and quantitated with ImageQuant software (Molecular Dynamics).

Construction of Reporter and Expression Plasmids—The Rf1-Pax-3 expression plasmid (28), obtained from George Chalepakis (here referred to as pCMV/Pax-3), was used for co-transfection experiments. A negative control expression plasmid, referred to as pCMV, was derived from pCMV/Pax-3 by excision of the Pax-3 cDNA fragment with *Bam*HI and *Xba*I. Blunt ends were formed using the Klenow fragment of DNA polymerase (New England Biolabs), electrophoresed on a 1.2% agarose gel, and linearized plasmid was excised from the gel and purified by Geneclean methods. The plasmid was circularized with T4 DNA Ligase.

For construction of reporter plasmids, double-stranded saab-1, saab-8, and saab-13 oligonucleotides (described above), as well as a PRS-9 control (AGCTTGATTAGCACCGTTACGGTCTAGAGGTCTAGC) were kinased with T4 polynucleotide kinase (New England Biolabs). The pTKGH reporter plasmid (HGH-TGES 100T kit, Nichols Institute), which contains herpes simplex virus thymidine kinase promoter sequences fused to the hGH (human growth hormone) gene, was digested with *Hin*dIII and dephosphorylated as described above. 50 ng of each double-stranded oligonucleotide was used in ligation reactions along with 100 ng of *Hin*dIII-digested dephosphorylated pTKGH. Individual clones were used in PCR reactions containing primers designed to the pTKGH plasmid, flanking the site of insertion, to verify the presence of insert. These forward and reverse primers were GTAAAACGACGGCCAGTG and TGAAGCAGGACCTGCAGC, respectively. The sequences of inserts were verified by sequencing with an upstream primer designed to the pTKGH vector, GCAAGGCGATTAAGTTGG. Recombinant reporter plasmids contained PRS-9, saab-1, and saab-13 inserts with the ATTA 5′ to the GTTAT element, and the saab-8 insert was found in the opposite orientation.

Transfections—NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10 mM HEPES, pH 7.4, 50 units/ml penicillin (Life Technologies, Inc.), 50 μg/ml Streptomycin (Life Technologies, Inc.), and 2 mM L-glutamine (Life Technologies, Inc.) and were grown in 5% CO_2 at 37 °C. Cells were plated in 35-mm tissue culture plates and transiently transfected when they were 60–70% confluent with a total of 30 μg of DNA by $CaPO_4$-mediated transfection, using the CellPhect transfection kit (Amersham Pharmacia Biotech). 10 μg of reporter plasmid, 10 μg of pCMV/Pax-3 or pCMV, and 10 μg of calf thymus DNA (Sigma) were used to transfect duplicate cultures, in three separate experiments. DNA precipitates were incubated with cells for 16 h, followed by removal of the precipitate and incubation for 1 h with fresh medium. This medium was again replaced, and cells were incubated for an additional 48 h. At the end of the culture period, media were collected for secreted growth hormone assays, using the HGH-TGES 100T radioimmunoassay kit (Nichols Institute), according to the manufacturer's suggested protocol. hGH standards, provided with the kit, were diluted with medium to determine a standard curve for each individual assay, to estimate actual amounts of hGH expression. Results of four repeated transfection experiments performed on successive days, each of which was performed in duplicate plates for each combination of transfected DNA, was analyzed statistically by analysis of variance, followed by *post hoc* Duncan's multiple range test, using a Statistica software application for the Macintosh.

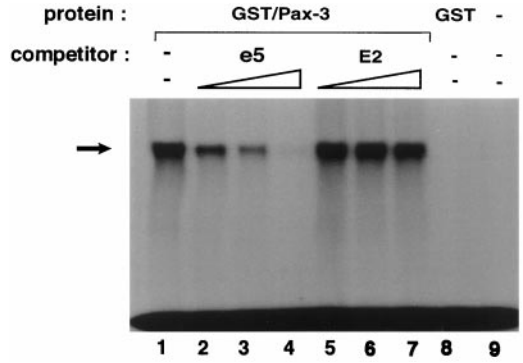

FIG. 1. **Gel mobility shift assay of binding reactions performed with GST/Pax-3 fusion protein or GST control protein, and radiolabeled e5 probe.** Reactions were performed in the absence or presence of 0 (*lanes 1* and *5*), 50- (*lanes 2* and *6*), 100- (*lanes 3* and *7*), or 200- (*lanes 4* and *8*) fold molar excess of unlabeled specific (*e5*), or nonspecific (*E2*), competitor. The specific shifted complex in indicated.

RESULTS

Selection of Pax-3 Binding Sequences among Random Oligonucleotides Using Both DNA-binding Domains of Pax-3—To express both of the Pax-3 DNA-binding domains for selection of novel binding sequences, a GST/Pax-3 fusion vector was constructed as described under "Experimental Procedures." This GST/Pax-3 fusion protein was able to bind to an oligonucleotide containing the *Drosophila* e5 sequence, a cognate Pax-3 binding element, as shown by gel mobility shift assay (Fig. 1). A single shifted complex was formed in the presence of the GST/Pax-3 fusion protein (Fig. 1, *lane 1*), and this complex was competed by 50–200-fold molar excess of unlabeled e5 oligonucleotide (Fig. 1, *lanes 2–4*). In contrast, a nonspecific oligonucleotide, derived from nt −30 to +40 of the adenovirus E2A promoter (E2), did not compete for binding (Fig. 1, *lanes 5–7*), demonstrating that the interaction between GST/Pax-3 and e5 was specific. The GST protein alone failed to form a complex with the e5 probe (Fig. 1, *lane 8*), demonstrating that the DNA-binding domains of Pax-3 were required for this interaction.

This GST/Pax-3 fusion protein was used in the selection of Pax-3 binding sites from a pool of random oligonucleotides using a modification of the "saab" (selected and amplified binding sites) procedure (35). The separation of GST/Pax-3-bound radiolabeled oligonucleotides from unbound oligonucleotides was accomplished by nondenaturing gel electrophoresis. A total of four rounds of selection and amplification were performed. No radiolabeled DNA was detected at the position that co-migrated with an e5 probe bound to GST/Pax-3 following the first round of

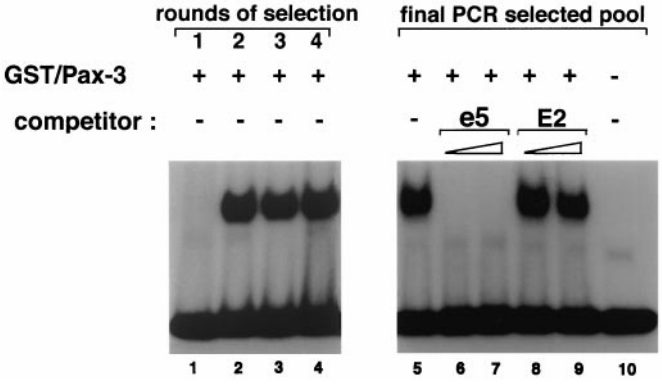

FIG. 2. **Random oligonucleotide binding site selection with GST/Pax-3.** A radiolabeled pool of oligonucleotides was incubated with the eluted GST/Pax-3 protein and reactions separated by nondenaturing gel electrophoresis as described under "Experimental Procedures." The shifted complex from each lane was excised, reamplified, and used in the subsequent binding reaction. After four rounds of selection, the enriched pool of oligonucleotides was tested for binding specificity by competition gel shift assays using 0 (*lane 5*), 50 (*lanes 6* and *8*), and 100 (*lanes 7* and *9*) ng of specific (*e5*) or nonspecific (*E2*) unlabeled competitor.

FIG. 3. **Alignment of sequences containing the "ATTA-N$_n$-GTTAT" consensus sequence for GST/Pax-3, where "*n*" represents a variable intervening spacer length between the two putative binding sites.** The ATTA and GTTAT of the derived consensus sequences are indicated in *bold*. The presence of an indentical spacer sequence in the two $n = 13$ clones could indicate that a single sequence was selected and that during subsequent PCR, a single base change (see "T" at position 2 of the top sequence and "A" at position 1 of the bottom sequence) occurred, resulting in two different clones containing an almost identical sequence.

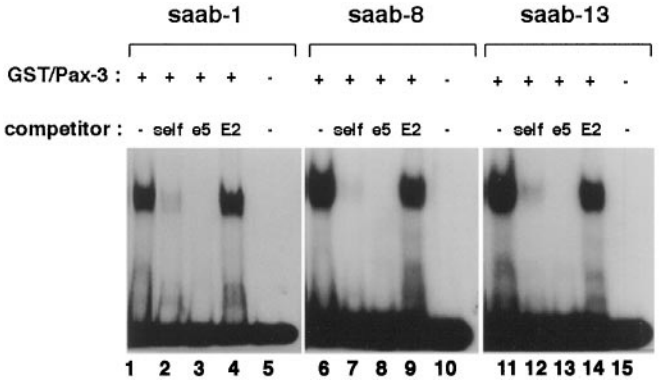

FIG. 4. **Binding specificity of subset of selected oligonucleotides.** Gel mobility shift assay of binding reactions contained radiolabeled probe made from each of three GST/Pax-3 selected oligonucleotides (saab-1, saab-8, and saab-13) and GST/Pax-3, in the presence of 0 (*lanes 1*, *6*, and *11*) or 250-fold molar excess of either unlabeled homologous competitor (*self*, *lanes 2*, *7*, and *12*), e5 (*lanes 3*, *8*, and *13*), or nonspecific E2 (*lanes 4*, *9*, and *14*) oligonucleotide.

selection (Fig. 2, *lane 1*), however, by the second round, a shifted complex was detected (Fig. 2, *lane 2*). The enriched pool of oligonucleotides that was obtained after the fourth round of selection was specifically competed with excess e5 competitor (Fig. 2, *lanes 6* and *7*), but not with the nonspecific E2 competitor (Fig. 2, *lanes 8* and *9*), demonstrating that GST/Pax-3 binds specifically to the pool of randomly selected oligonucleotides.

Sequence Analysis of GST/Pax-3 Selected Oligonucleotides—The enriched pool of GST/Pax-3 binding sequences was cloned into plasmid and sequenced. Of 47 independent clones that were sequenced, 45 were unique. All but two of these sequences contained at least one ATTA motif, the core homeodomain recognition sequence. In addition, all but one of the selected oligonucleotides contained a "GTNNN" motif, but none contained either the paired domain recognition sequence of e5 (GTTCC) or a base-substituted derivative of the paired domain binding site of e5, PRS-9 (GTTAC), to which Pax-3 can also bind (20). Instead, the sequence, GTTAT, which has not previously been identified in any Pax-3-binding elements, was present in 14 (33%) of the selected clones. Nine of the GTTAT-containing oligonucleotides, representing 20% of the 45 unique sequences, also contained an upstream ATTA. No other novel consensus element distinct from GTNNN was found among the selected oligonucleotides. Interestingly, the sequences between the ATTA and GTTAT were variable both in length (consisting of 1, 4, 5, 8, or 13 bases) and in base composition; the only apparent similarity of sequence on either side of the ATTA and GTTAT was an "A" or a "T." The selected oligonucleotides containing this consensus are shown in Fig. 3.

Binding Characteristics of Selected Sequences—Three of the nine sequences containing the consensus binding site were further evaluated. Specific probes were made for saab-1, saab-8, and saab-13, which contained 1, 8, or 13 bp between the ATTA and GTTAT elements, respectively (Fig. 3). Specificity of binding to GST/Pax-3 by each probe was tested in gel mobility shift assays, using homologous competitor oligonucleotides (Fig. 4). With each probe, a single complex was observed in the absence of competitor DNA, and this complex was competed by both the homologous (*lanes 2*, *7*, and *12*) and e5 oligonucleotides (*lanes 3*, *8*, and *13*), but not with the nonspecific E2 oligonucleotide (*lanes 4*, *9*, and *14*). These observations suggest that the interactions between each of the selected probes and GST/Pax-3 were specific.

Previous studies have indicated that oligonucleotides in which a paired domain and a homeodomain binding element were either very closely spaced or widely separated bind poorly or not at all (22). In addition, binding of many proteins to DNA is sensitive to the phasing around the DNA helix on which recognition elements are located (37, 38). Although Fig. 4 showed that each of the oligonucleotides with variable spacing could be bound by Pax-3, it was possible that each was bound with different affinities. To test this, binding competition analyses were performed with increasing amounts of homologous competitor for each of the e5, saab-1, saab-8, and saab-13 probes, and the concentration of competitor that inhibited binding by 50% was used to determine relative binding affinity (Fig. 5*A*). By this method, the affinity of GST/Pax-3 for e5 and saab-8 was equal, but it was reduced by 9- and 12-fold, respectively, for saab-1 and saab-13 (Fig. 5*B*). These results indicate that the tested oligonucleotides fall into at least two distinct categories of Pax-3 binding affinity: a higher affinity group, containing the e5 sequence and the saab-8 selected sequence, and a lower affinity group, containing the saab-1 and saab-13 sequences.

Effect of Sequence and Spacing of the DNA Consensus Sequence on Pax-3 Binding Affinity—The existence of at least two affinity classes of specific Pax-3 binding sites among the tested oligonucleotides prompted us to examine whether the variable length of the intervening spacer region was an important determinant for high affinity binding. To test this, four of the five nt between the core binding elements of the e5 oligonucleotide

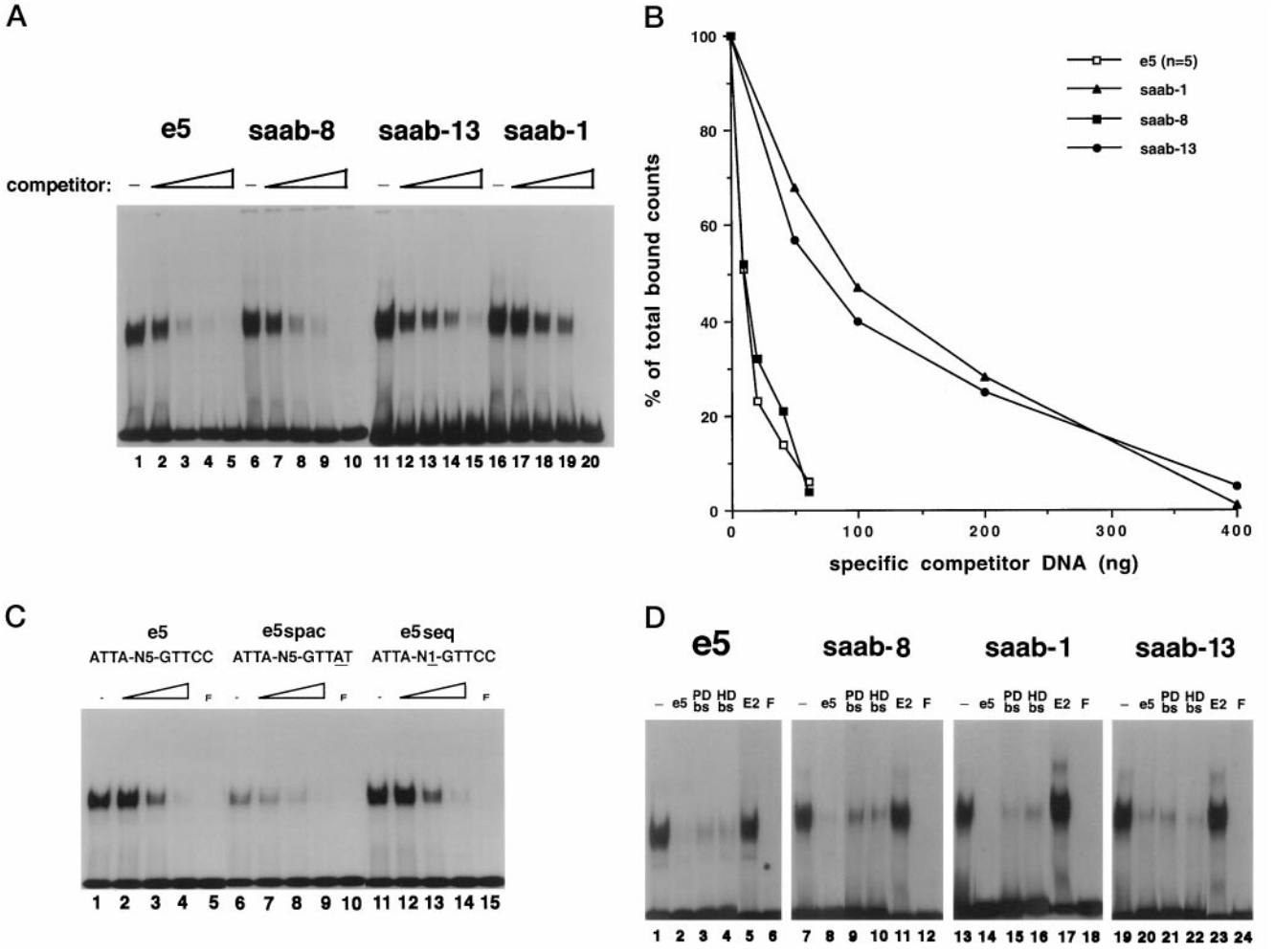

FIG. 5. **Relative affinities of selected oligonucleotides for GST/Pax-3.** *A*, gel mobility shift following binding reactions containing radiolabeled probes for e5, saab-8, saab-1, and saab-13, with GST/Pax-3 and either zero (*lanes 1*, *6*, *11*, and *16*) or increasing amounts of unlabeled homologous competitor. 150-, 300-, 600-, and 900-fold molar excess of homologous competitor were used for the e5 and saab-8 oligonucleotides, whereas 750-, 1500-, 3000-, and 6000-fold molar excess of homologous competitor was used for the saab-1 and saab-13 oligonucleotides. *B*, inhibition of Pax-3 binding by homologous competitor oligonucleotides. Quantitation of probe binding in the presence of increasing amounts of competitor from four representative binding reactions as shown in *A*, plotted as a percent of total binding in the absence of competitor. *C*, competition gel shift assay using 10 fmol of radiolabeled probes for the e5 (*lanes 1–5*), e5spac derivative (*lanes 6–10*), or the e5seq derivative (*lanes 11–15*), and GST/Pax-3, in the presence of zero (−) or 10-, 50-, and 100-fold molar excess of homologous competitor. *F*, free probe. *D*, competition gel shift assay using 10 fmol of radiolabeled probe for e5 (*lanes 1–6*), saab-8 (*lanes 7–12*), saab-1 (*lanes 13–18*), and saab-13 (*lanes 19–24*), with GST/Pax-3 and either 0 (−) or 200-fold molar excess of unlabeled competitor containing the e5 binding site (e5), the saab-8 oligonucleotide base substituted at the putative homeodomain binding site (*PDbs*), the saab-8 oligonucleotide base substituted at the putative paired domain binding site (*HDbs*), or the nonspecific E2 oligonucleotide. *F*, free probe.

were deleted, leaving a single "T," as found in the lower affinity saab-1 oligonucleotide. This mutation (e5spac) significantly reduced both the total binding capacity and binding affinity of e5 (Fig. 5*C*, *lanes 6–10*). Thus, the lower GST/Pax-3 binding affinity observed for saab-1 is likely because of constrained binding imposed when the two recognition sequences are placed only 1 bp apart from each other. Constrained binding when elements are too far apart may also be the explanation for the lower binding affinity for saab-13. The variation of the sequence of the presumed paired domain binding site, in contrast, did not appear to be responsible for the reduced affinity of the saab-1 or saab-13 oligonucleotides, because substitution of the "GTTCC" core paired domain binding sequence of e5 for GTTAT (mutation e5seq) had no apparent effect on binding affinity (Fig. 5*C*, *lanes 11–15*). This latter result was to be expected, because the presence of the GTTAT instead of GTTCC in the saab-8 oligonucleotide did not impair binding (Fig. 5*A*). Indeed, the similar affinities of GST/Pax-3 for e5 and saab-8, in which the core recognition elements are separated by 5 and 8 bp, respectively, suggests that spacing variation, even when the orientations of the putative contact sites for the paired and homeodomains are oriented differently by one-third of a turn around the DNA helix, is remarkably flexible.

Single-site Oligonucleotides Can Compete for Binding to Pax-3, but with Lower Affinity—To test whether both the ATTA and GTTAT were necessary for binding to GST/Pax-3, oligonucleotides were synthesized to contain base substitutions replacing either the ATTA or the GTTAT element of the high affinity saab-8 oligonucleotide, and these oligonucleotides were tested for their abilities to compete for binding to e5 or to each of the three saab probes. As shown in Fig. 5*D*, oligonucleotides in which either of these elements was mutated competed significantly for binding to each of the probes, but neither of the single site oligonucleotides competed as efficiently as excess e5 competitor that contains both Pax-3 binding elements. Thus, whereas both sites are necessary for optimal binding, either one or the other of the putative binding sites found in our saab oligonucleotides can be deleted without eliminating Pax-3 binding.

Regulation of Selected Binding Sites by Pax-3 during Transient Transfection—Previous studies have shown that Pax-3

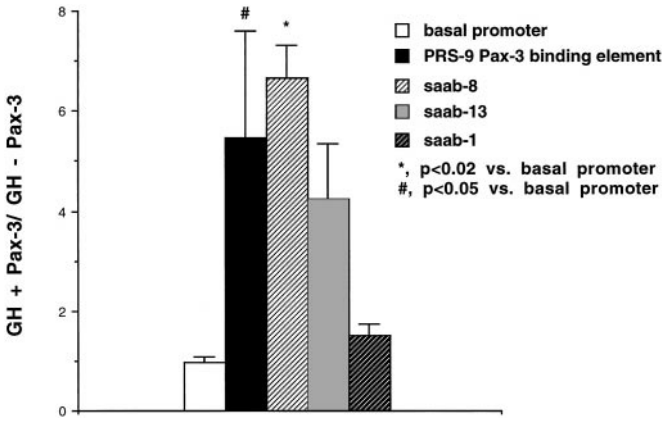

FIG. 6. **Activation of hGH expression by reporter plasmids containing Pax-3 binding sequences.** Four replicate transient transfection experiments of NIH-3T3 cells with indicated reporter constructs were co-transfected with either pCMV or pCMV/Pax-3 expression plasmids. -Fold activation of each reporter plasmid was determined from the ratio of hGH expressed by cultures co-transfected with pCMV-Pax-3 to those co-transfected with pCMV. *, $p < 0.02$, and #, $p < 0.05$, compared with plasmid with no Pax-3 binding elements.

will trans-activate reporter plasmids containing the e5 sequence (ATTA-N_5-GTTCC) or a base-substituted derivative of e5 (ATTA-N_5-GTTAC), called PRS-9 (24, 39, 40). To determine whether the *in vitro* selected saab Pax-3 binding sequences could be regulated by Pax-3 in living cells, each sequence was inserted into a pTKGH reporter plasmid, which contains the basal promoter element of the herpes virus thymidine kinase promoter linked to the human growth hormone reporter gene. A pTKGH plasmid containing the PRS-9 sequence was used as a positive control for Pax-3 trans-activation. PRS-9, rather than e5, was chosen as a control, because its paired domain binding sequence (GTTAC) more closely resembles the saab-selected GTTAT sequence than does the paired domain binding sequence of e5, and it has also been shown to be Pax-3-responsive in transfection assays (24). Reporter plasmids containing a single copy of either the PRS-9, saab-1, saab-8, or saab-13 sequence were generated and co-transfected into NIH-3T3 cells along with either a Pax-3 expression plasmid directed by the CMV promoter/enhancer sequences (pCMV/Pax-3) (28) or a control plasmid containing the CMV promoter/enhancer but lacking any Pax-3 cDNA sequence (pCMV). As shown in Fig. 6, expression of hGH from plasmids containing each of the tested inserts was increased upon co-transfection with pCMV/Pax-3, although to differing degrees. Only trans-activation of the plasmids containing the high affinity binding elements was statistically significant. In fact, the greatest trans-activation was observed using the saab-8-containing plasmid (13-fold), followed by PRS-9 (5.5-fold). Lower levels of activation were observed using the saab-13 sequence (3.9-fold) and the saab-1 sequence (1.7-fold), and these levels of activation were not significantly different from that of pTKGH. These results demonstrate that sequences that bind with higher affinity to Pax-3 mediate greater trans-activation by Pax-3 in living cells.

DISCUSSION

The purpose of this study was to search for optimal binding sequence(s) for Pax-3 using its combined paired domain and homeodomain. Identification of this sequence could then assist in the recognition of Pax-3 response elements in Pax-3-regulated genes. In the course of this work, we identified a consensus sequence that contains a previously unidentified paired domain recognition motif (GTTAT) located within 13 bp downstream of the known homeodomain recognition motif (ATTA). As well, we evaluated the effect of spacing of the ATTA and GTTAT on binding affinity and transcription regulation. The identification of GTTAT as a new paired domain recognition motif is noteworthy, because it is found in regulatory elements of at least two Pax-3-responsive genes, and trans-activation analysis demonstrated that it is even more responsive to Pax-3 than the previously studied PRS-9 element. When our results are taken in conjunction with the findings of others, in which Pax-3 can bind to an e5 variant containing the sequence, GTTAC (20), and the paired domain alone can bind to a GTCAC motif (23, 32), this suggests that the consensus element for the paired domain of Pax-3 can now be considered to be, GTPyA/CPy. The absence of any other completely novel paired domain consensus element among our saab-selected oligonucleotides would indicate that some variation of the GTPyA/CPy sequence is essential for binding by the Pax-3 paired domain.

Effect of Spacing between Homeodomain and Paired Domain Recognition Elements—GST/Pax-3 bound with similar affinities to the e5 element, in which the homeodomain and paired domain recognition sequences are separated by 5 nt, and to our saab-8 element, in which these elements are separated by 8 nt. The absence of an effect of different positions of the paired and homeodomain recognition elements around the DNA helix on binding affinity is quite unusual. Other regulatory proteins, for example, adenovirus E2F and λ repressors, must be localized at particular positions along the turns of the double helix in order for binding and regulation to occur (37, 38). One interpretation of these observations is that multimeric factors such as E2F and λ repressors assemble as complexes on DNA which are relatively inflexible, but a bipartite factor such as Pax-3, which binds as a monomer, can bend as both DNA-binding domains grasp their contact sites on the DNA. To our knowledge, this is an unprecedented characteristic of DNA-binding factors. When spacing requirements of POU proteins, another class of bipartite DNA-binding factors, which contain a POU domain and a POU-type homeodomain, has been examined, such flexibility has not been observed. For example, Brn-3, a member of the POU-IV class of transcription factors, will only bind to DNA when core motifs are separated by 3 nt (41). Brn-2, a member of the POU-III class of transcription factors, will tolerate spacing of the core motifs by either 0, 2, or 3 nt, but exhibits variable affinities for elements with differential spacing (41).

On the other hand, the ability of the Pax-3 DNA-binding domains to grasp its recognition elements at different locations does not appear to be unlimited, as reducing the distance between ATTA and GTTAT to 1 nt, or increasing it to 13 nt, markedly reduced binding affinity. Indeed, if the binding sequences are too close or too far apart from each other, simultaneous occupancy of DNA by both binding domains appears to be constrained, resulting in low affinity binding that was indistinguishable from binding to a single site and poor transcriptional responsiveness. Whereas others similarly found that when the paired domain and homeodomain recognition elements were separated by as few as 2 bp or as many as 15 bp, little or no binding occurred (22), our results indicate that weak binding can occur under such circumstances and might be associated with a modest transcriptional response.

Relationship of High and Low Affinity Binding to Regulation of Genes during Development—Although others have not detected binding of Pax-3 to either the homeodomain recognition sequence (20) or to the paired domain recognition sequence (30) in the absence of the other, our results, in which oligonucleotides containing only one or the other putative Pax-3 recognition sequence competed for binding to e5, demonstrated that the GST/Pax-3 fusion protein will recognize each individual binding element in the absence of the other. However, these interactions with only one recognition element appear to be of

low affinity, similar to those in which the recognition elements are either too close or too far apart.

Whether the presence of a single binding element is sufficient to activate transcription was not tested here. However, because neither of the low affinity saab-1 nor saab-13 elements were significantly trans-activated, it would be expected that genes associated with single elements would be weakly trans-activated as well. If this is the case, then it should be noted that almost all of the potential Pax-3 binding sequences found in the few identified Pax-3-responsive genes are low affinity. In the myelin basic protein promoter, there is one inverted GTTAT and two inverted GTTCC elements, but none of these elements is located in the proximity of a homeodomain binding element (42). Similarly, the human c-*MET* promoter contains one inverted GTTCC, and a similar element, GGTCC, but no nearby homeodomain binding element (43). The chicken *myoD* promoter, on the other hand, contains two inverted GTTAT elements located 15 and 18 nt upstream of inverted ATTA elements, three GTTCC elements located 1, 9, 12, or 15 nt downstream of ATTA elements, and four additional GTTAT or GTTCC elements located at least 200 bp away from homeodomain recognition elements. Because, based on the results presented here, only the GTTCC located within 9 nt of the ATTA of the *myoD* promoter is likely to bind Pax-3 with high affinity, it might be the only element that is significantly responsive to Pax-3 at the transcriptional level. Further identification of Pax-3-responsive genes and examination of effects of mutation of putative Pax-3 binding elements on transcriptional responsiveness will be necessary before the biological significance of these sequences in naturally occurring genes can be appreciated.

Although activation of the saab-13 element was not significantly greater than that of the basal TK promoter, it was also not significantly less than that of the PRS-9 element. Perhaps modest activation of genes associated with low affinity Pax-3 binding sites occurs when nuclear Pax-3 levels are very high. Likewise, because high affinity Pax-3 binding sites require a lower concentration of Pax-3 for stable binding, genes that possess these elements would be preferentially expressed when there are low levels of Pax-3 have accumulated in the cell. These may be the genes that are turned on first during the induction of *Pax-3* expression in a given embryonic tissue. This model is similar to that suggested for heat shock factor activation of heat shock response genes, whereby the preferential induction of the hsp 82 gene, over other heat shock response genes, appeared to be because of the higher affinity of heat shock factor for the binding site located upstream of the hsp 82 gene (44). Alternatively, it is intriguing to speculate that winding of DNA along histones could convert some low affinity elements into high affinity elements by arranging distant paired domain and homeodomain binding sites into close proximity to one another.

The sensitivity of genes associated with high and low affinity Pax-3 binding sites to relative nuclear levels of Pax-3 may help to explain how Pax-3 mutations lead to congenital malformations in mice and humans. For example, Pax-3 mutations in heterozygous *Splotch* mice cause only pigmentation defects, with apparently normal neural tube development. Thus, one might predict that the lower level of Pax-3 expression in heterozygotes is enough to activate high affinity response genes controlling neural tube development, but that the threshold of Pax-3 expression required for activation of lower affinity response genes in melanocyte precursors derived from the neural crest is not met. Similarly, maternal diabetes, which causes expression of *Pax-3* to be significantly reduced and is associated with increased neural tube defects (8), may lower the level of Pax-3 below a threshold required to activate the normal complement of Pax-3-responsive genes, with low affinity targets being particularly affected. Identification of additional Pax-3-responsive genes and their associated transcription control elements will be needed to more completely understand the functional significance of high and low affinity Pax-3 binding sites during embryonic development.

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Identification of a New Binding Motif for the Paired Domain of Pax-3 and Unusual Characteristics of Spacing of Bipartite Recognition Elements on Binding and Transcription Activation

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