Identification of Novel Pax-2 Binding Sites by Chromatin Precipitation*

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The Pax genes encode a family of developmental transcription factors that bind to specific DNA sequences via the paired domain and are necessary for the morphogenesis of a variety of tissues. The murine Pax-2 gene, through alternative splicing, encodes two nuclear proteins, Pax-2A and Pax-2B, which are transiently expressed during the differentiation of specific neural cell types and early kidney formation. In order to identify potential in vivo Pax-2 target sequences, chromatin from embryonic neural tube was immunoprecipitated with Pax-2 specific antibodies and cloned. Two unique immunoprecipitated clones containing three specific Pax-2 binding sites were identified by functional binding assays using Pax-2 proteins produced in both Escherichia coli and eukaryotic cells. In vitro DNA binding assays, using Pax-5 and Pax-8 DNA recognition sequences as well as the three immunopurified Pax-2 binding sites, demonstrated that both forms of the Pax-2 protein bind DNA with a similar specificity and that this binding is mediated by the paired domain. The binding sites identified in this report share significant homology among themselves and with previously defined consensus sequences for Pax-5 and Pax-2. The genomic clones can now be used as sequence tags to identify potential target loci.

The mammalian Pax gene family contains at least nine members that are expressed in distinct spatiotemporal patterns during embryogenesis and regulate the morphogenesis of a variety of tissues (1–4). As determined by the analysis of both naturally occurring and genetically engineered mutations, Pax proteins may function in the specification, adhesion, migration, or proliferation of progenitor cells in the developing nervous system (5, 6), vertebral column (7), kidney (8), eye (9), and immune system (10). The Pax genes encode transcription factors that bind as monomers to specific DNA sequences via the 128-amino acid paired domain. The paired domain, which is located at the amino terminus of the Pax proteins, is composed of two structurally independent subdomains, each of which contains a helix-turn-helix motif (11). The NH2- and COOH-terminal subdomains of the Pax-5 paired domain bind to adjacent major grooves of the DNA helix (12), while the Drosophila paired protein binds to one major groove through its NH2-terminal paired subdomain (11). The NH2-terminal subdomain also contains a β turn motif and COOH-terminal tail, which make contacts in the minor groove and along the phosphate backbone of the DNA. Alignment of the Pax binding sites indicates that the NH2-terminal subdomain, the sequence of which is highly conserved between the Pax proteins, contacts a conserved recognition sequence whereas the more divergent COOH-terminal subdomain appears to play a role in site-specific DNA recognition (12).

The murine Pax-2 gene encodes two nuclear proteins, Pax-2A and Pax-2B, which are transiently expressed during brain, neural tube, kidney, eye, and ear development (13–15). Partial loss of Pax-2 function in mice and humans results in kidney and retinal defects (16, 17). In addition, it appears that the transition of kidney mesenchyme to epithelium, its proliferation, and subsequent terminal differentiation requires the properly timed activation and repression of Pax-2 (8, 18). The expression data also suggest that Pax-2 may be involved in the differentiation of specific neural cell types within the hindbrain and spinal cord (19, 20). Several in vitro Pax-2A DNA recognition sequences have been identified based on PCR selection of Pax-2A bound random oligonucleotides (21), revealing a fairly divergent consensus type sequence. Pax-2 binding sites have also been deduced by random nucleotide substitutions within a core paired domain binding sequence originally defined for the Pax-1 protein (22). Although binding sequences and genes regulated by Pax-5 (12, 23–26), Pax-6 (27, 28), and Pax-8 (29) have been identified, there are currently no known in vivo DNA recognition sequences nor genes known to be regulated by Pax-2.

To better understand how Pax-2 exerts its morphologic function, it is necessary to identify in vivo DNA binding sites and genes activated or repressed by the Pax-2 protein. As a first step to isolate Pax-2 target genes in the developing spinal cord, DNA sequences bound to the Pax-2 protein were enriched from native chromatin, using Pax-2 specific antibodies, and cloned. Two unique genomic clones containing three specific Pax-2 binding sites were identified. The results demonstrate that both forms of the Pax-2 protein bind DNA with a similar specificity and that this binding is mediated by the paired domain. The report also demonstrates the feasibility of the chromatin immunoprecipitation method for the isolation of binding sites that can subsequently be used as sequence tags for the identification of loci regulated by transcription factors.

EXPERIMENTAL PROCEDURES

Pax-2 Antibodies—The polyclonal antibodies were generated against a glutathione fusion protein, containing amino acids 188–385 of the COOH-terminal domain of Pax-2, as described previously (15). The antibodies specifically recognize Pax-2A and Pax-2B on Western blots of embryonic kidney and neural tube (15, 19). The antibodies also recognize Pax-2 in the kidney and neural tube by immunocytochemistry on frozen sections of whole embryos (15, 19, 30). There is no cross-reactiv-
ity with Pax-8 in the kidney or thyroid as determined by immunocytochemistry and Western blotting (15).

Chromatin Immunoprecipitation—The general procedure was as described by Gould et al. (31) with the following modifications. To prepare nuclei, embryonic day 11 mouse spinal cords were isolated from single litters containing 10–12 embryos, washed in PBS, (pH 7.5), and resuspended in NB (10 mM Tris, pH 8.0, 0.5 mM DTT, 0.1% Triton X-100, 0.1 mM sucrose). The tissue was homogenized 20 times with a type A Dounce homogenizer and gently mixed with NB2 (NB1 with 0.25 mM sucrose). NBS (10 mM Tris, pH 8.0, 5 mM MgCl₂, 0.5 mM DTT, 0.33 mM sucrose) was layered under the cell suspension, and the nuclei were pelleted at 1000 × g for 5 min at 4°C. Typical yields were 0.5–1.0 × 10⁹ nuclei/experiment. Isolated nuclei were resuspended in 10 mM HEPES, pH 7.9, 10 mM KCi, 2 mM MgCl₂, 0.5 mM DTT plus protease inhibitors (1 mM APMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin) at a concentration of 1 × 10⁷/ml. The chromatin was digested in situ with 100–250 units/ml Sau3A for 30 min at 37°C. The nuclei were pelleted at 750 × g for 2 min, and resuspended in 1 ml of 12 mM Tris, pH 7.5, 3 mM EDTA plus protease inhibitors (as listed above), and slowly rotated at 4°C for 30–60 min. The nuclear debris was pelleted at 750 × g for 2 min, and the soluble chromatin remaining in the supernatant. The chromatin supernatant was transferred to a clean tube, and NaCl and BSA were added to final concentrations of 100 mM and 1 mg/ml, respectively. After a 5-min incubation, the supernatant was centrifuged at 12,000 × g for 5 min to remove any remaining precipitate.

The IgG fractions of anti-Pax-2 antisera were coupled to Amino- link-agarose beads (Pierce) according to manufacturer’s directions. Uncoupled and rabbit anti-Pax-2 IgG-coupled agarose beads were washed three times in 12 mM Tris, pH 7.5, 3 mM EDTA, 100 mM NaCl, and 1 mg/ml BSA. The chromatin supernatant was preabsorbed with the uncoupled agarose beads for 30 min at room temperature with slow rotation. The agarose was pelleted at 3000 × g for 30 s, and the supernatant was divided into two equal fractions. One half of supernatant was incubated with uncoupled agarose beads, and the other half was incubated with rabbit anti-Pax-2 IgG-coupled beads for 45 min at room temperature with slow rotation. The agarose was pelleted and washed three times with PBS containing 1 mg/ml BSA and 1 mM CaCl₂. The agarose was resuspended in protease buffer (50 mM Tris, pH 8.0, 1% SDS, 100 mM NaCl) containing 1 mg/ml Pronase and incubated for 60 min at 50°C. The DNA was extracted with phenol:chloroform (1:1), ethanol-predispended, and ligated into the BamHI site of pBlueScript (Bst) KS⁺ (Stratagene Inc.). GenBank (release 84.0) and EMBL (release 39.0) database searches were performed. The BESTFIT program was used for sequence alignments (Genetics Computer Group Sequence Analysis software package).

Pax-2 Protein Sources—Recombinant Pax-2 proteins were expressed in and purified from Escherichia coli using the pRSET protein expression system (Invitrogen). Ncol (blunt-ended) HindII fragments, encompassing the 5′- and 3′-flanking sequences of Pax-2, were inserted into the PvuII/HindIII sites of the T7 expression vector pRSET-A. Cleavage of the pRSET-A-Pax-2a plasmid with BamHI yielded a DNA fragment that included Pax-2 coding sequences up to, but not including, the octapeptide sequence. This BamHI fragment was inserted into the BamHI site of the pRSET-A vector to produce the pRSET-A-Pax-2a (PD) plasmid. The expression and purification of recombinant proteins were performed according to manufacturer’s protocol (Invitrogen). Briefly, recombinant Pax-2 proteins were expressed by infecting log phase, T7 polymerase-deficient E. coli (strain J109) with an M13 phage that contained the T7 RNA polymerase gene. The recombinant fusion proteins, which contain a hexahistidine nickel affinity motif within the NH₂-terminal fusion peptide, were bound to Ni²⁺-chelate resin (Invitrogen) with or without the addition of 0.5 M NaCl. The protein was eluted using a stepwise pH gradient. Proteins eluted from the metal affinity column were renatured by dialysis using a step gradient (8, 6, 4, 2, and 1 × 1 × 1 × 1 × 1) into Z-buffer (25 mM HEPES, pH 7.8, 20% glycerol, 12.5 mM MgCl₂, 0.1 mM KCl, 1 mM DTT) at 4°C. The yield of fusion protein from the metal affinity purification was 15–20 mg/liter of bacterial culture. On SDS-polyacrylamide gel electrophoresis, the predicted molecular masses of 55 kDa for Pax-2A, 53 kDa for Pax-2B, and 29 kDa for Pax-2 (PD). As a control, E. coli cells were transformed with the pRSET expression plasmid containing no insert, and bacterial proteins that bound and eluted from the metal affinity column served as control proteins in DNA binding assays.

Whole cell extracts containing full-length and truncated forms of the Pax-2 protein were prepared from transiently transfected COS-7 cells. Protein extracts were a gift from Martyn Goulding. COS-7 cells (5 × 10⁵) were transfected 4–6 h after seeding by the calcium phosphate method with 10 μg of pS1-Pax-2 expression plasmid and 10 μg of βts DNA/100-mm dish (32). As a control, COS-7 cells were mock-transfected or transfected with the βst or ps1 expression plasmid containing no insert. After a 16-h incubation with the calcium phosphate-precipitated DNA, the cell lysates were washed in PBS and refed with fresh medium. Cells were harvested 72 h post-transfection and resuspended in 200 μl of buffer (20 mM HEPES, pH 7.8, 150 mM NaCl, 25% glycerol, 0.5 mM DTT, 0.2 mM EDTA) plus protease inhibitors (0.5 mM APMSF, 0.5 μg/ml aprotinin, 2 μg/ml leupeptin). The cell lysate was sonicated three times for 5 s at high intensity and microcentrifuged at 12,000 × g for 10 min at 4°C. Western blot analysis using anti-Pax-2 antibodies of the cleared whole cell extracts using detected immunoreactive bands corresponding to the predicted molecular masses of 48 kDa for Pax-2A, 46 kDa for Pax-2B, 33 kDa for trPax-2A, and 31 kDa for trPax-2B within their respective protein extract. No immunoreactive bands were present in mock-, βst-, or pS1-transfected COS-7 cells. Pax-2 protein was immunoaffinity-purified from embryonic day 11 whole mouse nuclear extracts as described (15). The presence and relative size of the Pax-2 protein within the purified sample was confirmed by Western blot analysis using anti-Pax-2 antibodies (15). Chloroform acetyltransferase (CAT) assays were performed as described by Gorman et al. (32).

Cell Mobility Shifts/Supershifts/Competitions—The sequences of the H2A 2.2 (33) and Tg (29) oligonucleotides have been previously described. The sequences of the Pax-2 binding sites were as follows: P2BS1, 5′- TTCGTTGCTATGTAAGTTTGTTCCCTTGGAGATGT; P2BS2, 5′- TCGACTAGGAGCAATTAGTGCGAGCTGAAACCACACACATC.

Double-stranded H2A 2.2 and Tg oligonucleotides (25–100 ng) were end-labeled with T4 polynucleotide kinase and [³²P]ATP (3000 Ci/mmol). Double-stranded P2BS1 and P2BS2 oligonucleotides (25–100 ng) and gel-purified DNA fragments were labeled with Klenow enzyme and [³²P]dCTP or [³²P]dATP (3000 Ci/mmol). Binding reactions were performed in a total volume of 10 μl at 4°C for 30 min and contained 2–3 μl of immunoaffinity-purified Pax-2 protein or an empirically determined amount of purified bacterial Pax-2 fusion protein, 100 ng of poly(d-dC), ³²P-labeled probe (30,000–40,000 cpm), 0.5 mg/ml BSA, and Z-buffer. Binding reactions containing protein extracts prepared from transfected COS-7 cells were performed in a total volume of 15 μl at room temperature for 30 min and contained 5 μl of protein extract, 1 μg of poly(d-dC), binding buffer (15 mM Tris, pH 7.5, 90 mM KCl, 0.7 mM EDTA, 1 mg/ml BSA, 0.2 mM DTT, 1 mg/ml BSA, 0.2 mM DTT, 1 mg/ml BSA, 0.2 mM DTT, 1 mg/ml BSA, 0.2 mM DTT), 0.5% SDS, 0.5% sodium deoxycholate, and 5% (v/v) 10% glycerol. After competition experiments with purified bacterial Pax-2 protein were preincubated with the indicated weight excess of unlabelled DNA or poly(d-dC) for 10 min before adding the ³²P-labeled probe. For antibody supershift assays, protein A-purified rabbit anti-Pax-2 IgG or rabbit anti-laminin IgG were incubated at 80 μg/ml with purified bacterial Pax-2 protein, 500 ng of poly(d-dC), and ³²P-labeled probe. The laminin polyclonal antibody reacts with all three laminin chains and was kindly provided by H. Kleinman. Free DNA and DNA-protein complexes were resolved at room temperature on 6% neutral polyacrylamide gels in 0.5 × TBE at 150 V.

DNAse I Footprinting—DNAse I footprinting experiments were performed as described (30) with the following modifications. Increasing amounts of purified bacterial or recombinant Pax-2 protein were preincubated with 100 ng of poly(d-dC) for 10 min on ice. DNA probe (10,000 cpm) was added, and incubation was continued for 10 min on ice and then 5 min at room temperature. The DNA was cleaved with 0.1 units of RNase-free DNAse I for 30 s at room temperature, and the reaction was terminated. Guanine chemical cleavage reactions of the same DNA samples were performed with piperidine as described by Maxam and Gilbert (34).

RESULTS

DNA Binding Properties of the Recombinant Pax-2 Protein—In order to screen a potentially large number of clones that may contain binding sites, it was necessary to generate a large amount of biologically active Pax-2 protein. Both Pax-2A and Pax-2B proteins were expressed in E. coli and eukaryotic cells.
from transfected COS-7 cells as indicated. Tg(E) probes bound to whole cell extracts D, gel motility assay using H2A 2.2 (or immunopurified Pax-2 (mPax-2). recombinant Pax-2 proteins (as indicated) as probes bound to C the Tg sequence (s) using the H2A 2.2 sequence (B) or the Tg sequence (C) as probes bound to recombinant Pax-2 proteins (as indicated) or immunopurified Pax-2 (mPax-2). D and E, gel motility assay using H2A 2.2 (D) or Tg (E) probes bound to whole cell extracts from transfected COS-7 cells as indicated.

(Fig. 1). In addition, a number of useful truncated Pax-2 proteins were also expressed. The DNA binding properties of the Pax-2 protein were analyzed by gel mobility shift assays using two characterized recognition sequences, H2A 2.2 (32) and Tg (29), that are known to bind Pax-5 and Pax-8, respectively. The H2A 2.2 sequence is present within the promoter of the sea urchin H2A 2.2 late histone gene (32). The Tg sequence is present within the promoter of the murine thyroglobulin gene (29). The paired domains of Pax-2, Pax-5, and Pax-8 are 96–97% homologous (1). Based on this high degree of homology within the paired domain, it was proposed that these three proteins could recognize similar DNA sequences.

As expected, recombinant Pax-2A, Pax-2B, and Pax-2 (PD) fusion proteins purified from E. coli bound to the H2A 2.2 and Tg sequences (Fig. 1, B and C). Consistent with the smaller size of the Pax-2 (PD)-protein, the migration of the Pax-2 (PD)-DNA complex was faster than the migration of the Pax-2A and Pax-2B-DNA complexes. The presence of Pax-2A and Pax-2B protein within the retarded protein-DNA complexes was confirmed by gel mobility supershift assays using anti-Pax-2 antibodies (data not shown). There were no retarded protein-DNA complexes when the DNA probes were incubated with BSA or bacterial control proteins. To confirm these results, Pax-2A and Pax-2B proteins transiently expressed in COS-7 cells and immunopurifed mouse Pax-2 protein from nuclear extracts (mPax-2) also bound to the H2A 2.2 and Tg paired domain recognition sequences (Fig. 1, B and C). Consistent with the smaller size of the truncated Pax-2 proteins (trPax-2), the migration of the trPax-2 protein-DNA complexes were faster than the migration of the full-length Pax-2 protein-DNA complexes. Band shifts corresponding to Pax-2 protein-DNA complexes were not present when the probes were incubated with extracts prepared from mock- or Bst-transfected COS-7 cells. Finally, the bacterially expressed Pax-2A and Pax-2B protein-DNA complexes co-migrated with the mPax-2 shifted band (Fig. 1, B and C). These results demonstrate that the Pax-2 paired domain is sufficient for DNA binding, that both forms of the Pax-2 protein can independently bind DNA, and that bacterially expressed Pax-2 binds with similar properties as Pax-2 purified from embryonic nuclear extracts.

Isolation of DNA Clones Containing Pax-2 Binding Sites—To identify in vivo Pax-2 DNA recognition sequences, chromatin was prepared from nuclei of embryonic day 11 neural tube and solubilized by digestion with the restriction endonuclease Sau3A in situ, and Pax-2 protein-DNA complexes were immunoprecipitated with Pax-2 specific antibodies and cloned. Because the restriction enzyme digestion was done with intact nuclei, only transcriptionally active regions of the chromatin should be susceptible to endonuclease digestion. The chromatin was isolated from nuclei under low salt conditions in order to preserve protein-DNA complexes. As a negative control, half of the chromatin was immunoprecipitated with agarose beads alone or with preimmune IgG coupled to agarose beads. Two independent chromatin immunoprecipitation experiments were performed. In the first experiment, 18 Pax-2-enriched clones encompassing 11 kb of DNA were isolated with biotinylated Pax-2 antibodies and streptavidin-agarose beads. Ten control clones encompassing 10 kb of DNA and 40 Pax-2-enriched clones encompassing 26 kb of DNA were isolated from the second experiment in which DNA clones were immunoprecipitated with anti-Pax-2 antibodies directly coupled to agarose beads. The sizes of the DNA inserts ranged from 0.2 to 3.5 kb.

Immunopurified DNA clones were screened for the presence of a Pax-2 binding site by gel shift assays using recombinant Pax-2 proteins produced in E. coli. Plasmids containing DNA inserts were pooled together into groups of 3–5 plasmids such that the sizes of the DNA inserts within a group were different. The insert DNA was released from the Bst plasmid by endonuclease digestion and end-labeled, and the pooled clones were subjected to gel mobility shift assays in the presence of BSA or partially purified bacterially expressed Pax-2 protein (Fig. 2). Two Pax-2-enriched clones from the first experiment, four Pax-2-enriched clones from the second experiment, and one control clone displayed a retarded complex in the presence of Pax-2 protein. Further gel shift analysis of clones 2-10 and control clone 3-1, which were repetitive sequences, revealed that a bacterial protein present within the partially purified Pax-2 protein sample and not Pax-2 was binding to these sequences (data not shown). The binding of Pax-2 protein to four of the remaining Pax-2-enriched clones 2-4, 2-5, 2-6, and 2-17 was characterized in more detail.

Mapping of Pax-2 Binding Sites—DNA sequencing revealed that 2-5 and 2-6 were identical 620-base pair clones with no homology to any known sequences (hereafter referred to as...
pooled DNA sequences were incubated with BSA (control clone 3-1; amide gel. DNA inserts that displayed a band shift are indicated. Arrowheads: Pax-2A and Pax-2B protein-DNA complexes were supershifted in the presence of anti-Pax-2 antibodies. In the absence of antibodies, band shifts were present when Pax-2A and Pax-2B protein were incubated with the P2BS1 (Fig. 4A) and P2BS2 (Fig. 4B) sequences. These band shifts were not present when the sequences were incubated with BSA or control proteins. In the presence of anti-Pax-2 antibodies, the Pax-2A and Pax-2B protein-DNA complexes were supershifted (arrowheads) with most of the antibody-protein-DNA complexes precipitating in the wells of the gel (arrows). Anti-laminin antibodies did not affect the migration of the retarded complexes. These results were consistent with the immunoprecipitation of clone 2-6 with anti-Pax-2 antibodies from embryonic neural tube chromatin.

The ability of Pax-2 protein produced in eukaryotic cells to bind to the P2BS1 and P2BS2 sequences was also examined (Fig. 5). Gel mobility shifts were evident when P2BS1 (Fig. 5A) and P2BS2 (Fig. 5B) were incubated with full-length and truncated forms of the Pax-2 protein transiently expressed in COS-7 cells. These retarded complexes were not present when the probes were incubated with extracts prepared from mock- or Bst-transfected COS-7 cells. The same protein/DNA complex was also present when P2BS2 and a restriction fragment spanning nucleotides 189–276 of clone 2-6 (data not shown) were incubated with mPax-2.

To test for specificity of Pax-2 binding, the P2BS1 and P2BS2 sequences were incubated in the presence of increasing amounts of unlabeled competitor DNA (Fig. 6). In the absence of competitor DNA, band shifts were present when P2A-A and Pax-2B protein were incubated with the P2BS1 (Fig. 6A) and P2BS2 (Fig. 6B) sequences. The observed P2A-A and Pax-2B protein-DNA complexes were competed for completely by the addition of a 100-fold molar excess of unlabeled P2BS1 and P2BS2 oligonucleotides. In contrast, increasing amounts of the nonspecific competitor poly(dl-dC) did not reduce the amount of Pax-2 protein-DNA complex formation. These results confirmed that P2A-A and Pax-2B were specifically binding to the P2BS1 and P2BS2 sequences.

To confirm that a Pax-2 DNA recognition sequence was present within clone 2-17, a restriction fragment spanning nucleotides 55–106 (P2BS3) was subjected to gel shift assays (Fig. 7). Band shifts were present when this DNA probe was incubated with full-length Pax-2 proteins expressed in bacteria (Fig. 7A) and eukaryotic cells (Fig. 7B). Full-length Pax-2 protein-DNA complexes were not present when the DNA probe was incubated with BSA, control, mock, or Bst protein samples. pS1-trPax-2-A and pS1-trPax-2B-protein-DNA complexes could not be definitively identified, most likely due to their comigration with other protein-DNA complexes (Fig. 7B, arrow). Protein-DNA complex I did not contain Pax-2 protein, as it was not supershifted with anti-Pax-2 antibodies (data not shown), the addition of the H2A 2.2 oligonucleotide to the binding reaction did not reduce complex I formation (Fig. 7C), and the addition of cold clone 2-10 sequences to the binding reaction reduced the formation of complex I but did not affect the binding of Pax-2B (Fig. 7). Furthermore, following the addition of cold clone 2-10 sequences to the binding reaction, the Pax-2 (PD) protein-DNA complex was more visible (data not shown). The specificity of Pax-2 binding to this sequence was examined by competition gel mobility shift assays using bacterially expressed Pax-2B protein that was preincubated with a 300-fold molar excess of cold clone 2-10 sequences. A 100-fold excess of the nonspecific competitor (poly(dl-dC)) did not interfere with the binding of Pax-2B to the DNA probe, whereas a 100-fold excess of the specific (2-17 or H2A 2.2) dramatically decreased Pax-2B protein-DNA complex formation (Fig. 7C). These results confirmed that at least one specific Pax-2 binding site was present within clone 2-17 between nucleotides 55 and 106.

The nucleotide sequences of the three Pax-2 binding sites characterized in this report are compared in Fig. 8. There is significant homology over a 23-nucleotide stretch that spans the binding region. The previously reported consensus sequence derived by PCR amplification of random oligonucleotides bound to the Pax-2 paired domain also exhibits homology to the P2BS site, particularly the TCA nucleotide motif at the
The Pax-5 binding sequence is more loosely defined (12) does share features with the Pax-2 consensus consistent with the high level of homology between the Pax-2 and Pax-5 paired domains.

Comparison of Pax-2, Pax-5, and Pax-8 Binding to P2BS1 and P2BS2—Because of the homology both within the paired domain and in the COOH-terminal part of the Pax-2, Pax-5, and Pax-8 proteins, it is possible that the Pax-2 DNA binding sites may actually be bound by Pax-5 and Pax-8. The anti-Pax-2 antibody used for immunoprecipitation was made against the COOH-terminal amino acids 188–385 (15), spanning a region that is 49% and 35% identical to Pax-5 and Pax-8, respectively. The specificity of the Pax-2 antibodies has been demonstrated previously by Western blotting experiments (15) and immunocytochemistry (15, 19, 30). To further address potential cross-reactivity, the antibodies were tested against whole cell lysates made from Pax-2-, Pax-5-, and Pax-8-transfected cells (Fig. 9). Cells in 10-cm dishes were co-transfected with 16 μg of Pax expression vectors and the 2 μg RSV CAT plasmid as an internal standard. The whole cell lysates had near equal amounts of CAT activity, indicating similar transfection efficiencies (data not shown). In this assay, the antibodies do show some cross-reactivity to the Pax-5 protein but not to Pax-8 (Fig. 9A). However, the level of antibody reactivity to Pax-5 is significantly less than to Pax-2.

The transfected cell lysates were also used to determine if...
The identification of target DNA binding sites and associated genes is a necessary step toward elucidating the molecular mechanisms underlying Pax-2 function during development. We have demonstrated the feasibility of using specific antibodies and a functional DNA binding assay to screen for Pax-2 binding sites within the genome of expressing cells. Chromatin from embryonic neural tube was immunoprecipitated with Pax-2 specific antibodies and the enriched DNA cloned. Two unique immunoprecipitated DNA clones containing three Pax-2 binding sites were identified and confirmed by gel mobility shift assays using immunopurified Pax-2 and Pax-2 transiently produced in COS-7 cells. Specificity of binding was further confirmed by competition gel mobility shift assays and supershift assays using antibodies specific for the Pax-2 protein. The in vitro DNA binding data strongly suggest that these binding sites represent biologically relevant Pax-2 recognition sequences. A comparison of the three Pax-2 binding sites revealed a 23-base pair region that displays extensive homology. The Pax-2 consensus sequence derived from these three binding sites is similar to the previous Pax-2 (21) and Pax-5 (12) consensus recognition sequences. It is striking that Pax binding sites reflect an unusually high level of divergence making consensus site calculation difficult. This may be in part due to the large region of contact. The presence of a helix-turn-helix homeodomain at the COOH-terminal end of some Pax proteins (Pax-3, Pax-7, Prd) may also introduce additional specificity by stabilizing protein-DNA interactions through contact of neighboring homeodomain recognition sites (35, 36).

To study the DNA binding properties of the Pax-2 protein, recombinant Pax-2A, Pax-2B, and Pax-2 paired domain proteins, purified from bacterial cells, were compared to Pax-2 proteins synthesized by transfected eukaryotic cells and to immunoaffinity-purified Pax-2 from neural tube nuclear extracts. Regardless of the source, all Pax-2 proteins, including the paired domain only truncation, specifically bound to the H2A2.2 and H2A2.2 and Tg recognition sequences. A lack of post-translational modifications and the low renaturation efficiency of de-natured proteins to a biologically active form are problems associated with the production of recombinant proteins in E. coli (37). The isolation of Pax-2 protein from an endogenous source ensures that post-translational modification and folding of the protein is correct. In addition, any associated proteins that are needed for or enhance binding could co-purify along with the Pax-2 protein. The different isoforms and truncated forms of Pax-2 were also expressed in the COS-7 eukaryotic cell line. The binding of all three protein sources to the H2A2.2 and Tg paired domain DNA recognition sequences demonstrates that Pax-2 does not require specific post-translational modifications or cofactors for binding in vitro. In addition, the comigration of immunoaffinity-purified Pax-2 protein-DNA complexes with the bacterially expressed Pax-2 protein-DNA complexes suggest that Pax-2 is not binding to these sequences as a complex of proteins. Finally, the DNA binding ability of the bacterially expressed Pax-2 proteins demonstrates that the DNA binding domain of the proteins can properly refold following purification under denaturing conditions. However, the large molar excess of bacterially expressed proteins within the DNA binding assays suggest that only a small fraction of the solubilized protein is biologically active and/or that the lack of post-translational modifications affects the DNA binding affinity of these proteins. Despite these problems, the bacterially expressed Pax-2 proteins served as an abundant source of purified biologically active Pax-2 protein.
Alternative splicing of transcription factor gene transcripts can generate protein variants with diverse function (38, 39). For example, alternative splicing of the Pax-6 gene in vertebrates gives rise to a Pax-6 isoform with a 14-amino acid insertion in the paired domain that results in a dramatically altered DNA binding specificity (40). This alternatively spliced Pax-6 exon is lacking in the Drosophila (41) and sea urchin Pax-6 genes (42). In addition, alternative splicing of the murine and human Pax-8 gene gives rise to several Pax-8 isoforms that differ in their carboxyl-terminal regions downstream of the paired domain (43, 44). The DNA binding and transactivation properties of the Pax-8A and Pax-8B isoforms are similar to each other but differ from Pax-8C and Pax-8D. The results presented in this paper demonstrate that the Pax-2A and Pax-2B proteins have similar DNA binding specificity in vitro. In addition, truncated forms of the Pax-2 protein did not exhibit an altered DNA binding specificity compared to full-length Pax-2 proteins, which suggests that binding specificity is determined by the paired domain and that the 23-amino acid insertion does not affect the DNA binding properties of the Pax-2 protein. In addition, similar to Pax-8A and Pax-8B, there is evidence that Pax-2A and Pax-2B are transcriptional activators in cell culture assays (22). Moreover, in zebrafish both the length and sequence of the insertion are different from that of mouse Pax-2 (45). The analysis of this alternatively spliced Pax-2 exon from other species may help define the functional properties of the Pax-2A and Pax-2B proteins. Finally, a second alternative splice site has been identified in the murine and human Pax-2 gene that generates a protein with a serine/threonine/proline-rich COOH terminus (46). The DNA binding and transactivation properties of the resulting proteins are currently unknown.

Several lines of evidence suggest that Pax-2, Pax-5, and Pax-8 subfamily may serve the same redundant function in the neural tube where they are coexpressed in similar dorso-ventral patterns (3). The binding of the Pax-2 protein to Pax-5 (H2A 2.2) and Pax-8 (Tg) DNA recognition sequence and the ability of Pax-2 and Pax-8 to activate transcription from the same reporter construct in transient assays (43) support this hypothesis. Alternative splicing of Pax-2 and Pax-8 generates two proteins, Pax-2B and Pax-8B, which are collinear with Pax-5 along the less conserved COOH-terminal region of the proteins. Finally, Pax-5 mutant mice do not display any obvious neural tube abnormalities posterior to the hindbrain (10). It would be interesting to determine if Pax-2 or Pax-8, which are expressed at the midbrain-hindbrain junction, can rescue the abnormal development of the posterior midbrain and anterior cerebellum of Pax-5 mutant mice. Despite the high degree of homology within their paired domains, the binding sites identified in this report were able to distinguish among the Pax-2, 5, and 8 proteins. Pax-2 bound the P2BS1 and P2BS2 sequences with higher efficiency, compared to Pax-5 and Pax-8. The sites identified in this report probably represent only a small fraction of total available Pax-2 binding sites, and their biological relevance remains to be determined. The Pax-2 binding sites present in clones 2-6 and 2-17 were not able to confer Pax-2-dependent transcription activation in a transient transfection assay of P19 embryonal carcinoma cells. The Pax-2 binding regions were cloned upstream of the herpes simplex virus thymidine kinase promoter and the CAT gene as a reporter construct. The reporter was co-transfected with increasing amounts of Pax-2 expressing plasmid. However, increasing in CAT activity was not observed using either DNAs from clone 2-6 or 2-17. One possible explanation for these results is that specific cofactors required for Pax-2 trans-regulation of the immunopurified binding sites are lacking in P19 cells, as transactivation is dependent upon the cell line (22, 43). Furthermore, Pax-2-dependent transactivation in tissue culture requires multimerized binding sites, usually six, upstream of a basal level promoter. These multiple Pax binding sites are not found in genes known to be regulated by Pax-8 (29) or Pax-6 (27, 28). However, it cannot be ruled out that the orientation and/or accessibility of the binding sites required for Pax-2 trans-regulation in these assays is sub-optimal. Pax protein function may also depend on the context of the recognition sequence. For example, in developing B-lymphocytes, the Pax-5 protein acts as a transcriptional activator of the CD19 and VpreB genes, but is a repressor of the Ig3 enhancer (23–26). These results suggest that transcriptional regulation by the Pax proteins is complex and may be modulated by interactions with other proteins. It is also possible that Pax-2 may be acting as a repressor when bound to these sites by sterically hindering activating factors from binding either directly or by changing the conformation of adjacent DNA sequences (47). That the Pax-5 binding sites within the switch regions of the immunoglobulin heavy chain gene locus appear to function in heavy chain switch recombination suggest additional functions for Pax proteins independent of transcription regulation (48–51). In addition, the Drosophila homeodomain-containing proteins, Even Skipped and Fushi Tarazu, bind at low but significant levels to genes that are not expected to regulate (52). Future studies aimed at the identification of adjacent genes may elucidate the biological significance of these Pax-2 binding sites.

Several methods have been used for the isolation and identification of downstream target genes for transcription factors, one of which is the chromatin immunoprecipitation technique. This technique can enrich for genomic fragments that are bound by the protein of interest in vivo. The restriction enzyme, Sau3A, was used to digest the chromatin in intact nuclei. Thus, only accessible chromatin will be cut leaving much of the transcriptionally inactive chromatin too large to diffuse out of the nuclei. The low salt elution conditions favor the maintenance of protein-DNA complexes during immunoprecipitation with specific antibodies (30). Target genes for the Drosophila Ultrathorax homeodomain-containing protein (53, 54) and the thyroid hormone receptor have been identified through this technique (55). While the immunoprecipitation conditions favor the maintenance of protein-DNA complex associations, the stability of the complexes during the procedure has not been directly examined. To preserve protein-DNA complexes during immunopurification, the proteins can be cross-linked to the DNA with ultraviolet light or chemically before immunoprecipitation. Using cross-linked protein-DNA complexes, in vivo target genes have been immunopurified for the Ultrathorax protein (56) and the mammalian Hox-C8 homeodomain-containing protein (57) and in vivo DNA target sequences have been immunopurified for the Drosophila Polycomb protein (58). Approximately 10 fold enrichment for specific in vivo binding sites have been obtained through the noncross-linked procedure compared to over 100 fold enrichment for the chemically cross-linked technique. However, the cloning efficiency of the cross-linked immunopurified DNA is poor. In conclusion, through the identification of Pax-2 DNA recognition sequences, these studies have begun to address the function of Pax-2 during spinal cord development. Genomic DNA clones 2-6 and 2-17, which contain Pax-2 binding sites, can ultimately be used as molecular tags to identify nearby genes that may be regulated by Pax-2.

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REFERENCES
1. Walther, C., Guenet, J., Simon, D., Deutsch, U., Jostes, B., Goulding, M. D., Plachov, D., Balling, R., and Gruss, P. (1991) Genomics 10, 424–434.
2. Chalepakis, G., Stoykova, A., Wij holdhs, J., Tremblay, P., and Gruss, P. (1993) J. Neurobiol. 24, 1367–1384.
3. Stuart, E. T., Klous, C., and Gruss, P. (1993) Annu. Rev. Genet. 27, 219–236.
4. Read, A. P., and van Heyningen, V. (1994) Semin. Dev. Biol. 5, 323–332.
5. Epstein, D. J., Vekemans, V., and Gros, P. (1991) Cell 67, 767–774.
6. Tassabehji, M., Read, A. P., Newton, V. E., Harris, R., Balling, R., Gruss, P., and Strachan, T. (1992) Nature 355, 635–636.
7. Bailing, R., Deutsch, U., and Gruss, P. (1998) Cell 95, 531–535.
8. Rothenpieler, U. W., and Dressler, G. R. (1993) Development 119, 711–720.
9. Hill, R. E., Favor, J., Cai, J., Glaser, T., Jepeal, L., Walton, D. S., and Maas, R. L. (1994) Mol. Cell. Biol. 14, 381–391.
10. Kozmik, Z., Wang, S., Dorfler, P., Adams, B., and Busslinger, M. (1992) Mol. Cell. Biol. 12, 2662–2672.
11. Okabe, T., Watanabe, T., and Kudo, A. (1992) Eur. J. Immunol. 22, 37–43.
12. Singh, M., and Birshstein, B. K. (1993) Mol. Cell. Biol. 13, 3611–3622.
13. Neurath, M. F., Strober, W., and Wakatsuki, Y. (1994) J. Immunol. 153, 730–742.
14. Cvekl, A., Sax, C. M., Li, X., McDermott, J. B., and Piatigorsky, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4681–4685.
15. Holst, B. D., Goomer, R. S., Wood, I. C., Edelman, G. M., and Jones, F. S. (1994) J. Biol. Chem. 269, 22245–22252.
16. Zannini, M., Francis-Lang, H., Plachov, D., and Di Lauro, R. (1992) Mol. Cell. Biol. 12, 4320–4421.
17. Ryan, G., Steele-Perrins, V., Morris, J. F., Rauscher, F. J., III, and Dressler, G. R. (1995) Development 121, 867–875.
18. Gould, A. P., Broockman, J. J., Strutt, D. I., and White, R. A. H. (1990) Nature 348, 308–312.
19. Gorman, C. M., Mcfcat, L. F., and Howard, B. H. (1982) Mol. Cell. Biol. 2, 1044–1051.
20. Adams, B., Dorfler, P., Aguzzi, A., Kozmik, Z., Urbanek, P., Maurer-Fogy, I., and Busslinger, M. (1992) Genes & Dev. 6, 1589–1607.
21. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.
22. Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J., and Gruss, P. (1991) EMBO J. 10, 1135–1147.
23. Treisman, J., Harris, E., and Desplan, C. (1991) Genes & Dev. 5, 594–604.
24. Sharma, S. K. (1986) Sep. Sci. Tech. 21, 701–726.
25. Smith, C. W. J., Paton, J. G., and Nadal-Ginard, B. (1989) Annu. Rev. Genet. 23, 527–577.
26. Foulkes, N. S., and Sassone-Corsi, P. (1992) Cell 68, 411–414.
27. Epstein, J. A., Glaser, T., Cai, J., Jepeal, L., Walton, D. S., and Maas, R. L. (1994) Genes & Dev. 8, 2022–2034.
28. Quiring, R., Waldorf, U., Kloter, U., and Gehring, W. (1994) Science 265, 785–789.
29. Cvekl, A., and Busslinger, M. (1995) Mol. Cell. Biol. 15, 2858–2871.
30. Papi, T., Kozmik, Z., Kurnzaur, R., Dorfler, P., and Busslinger, M. (1993) Mol. Cell. Biol. 13, 6024–6035.
31. Polev, A., Wendler, F., Fickenscher, H., Zannini, M. S., Yaginuma, K., Abbott, C., and Plachov, D. (1995) Eur. J. Biochem. 228, 899–911.
32. Krauss, S., Schanssen, T., Korzh, V., and Fjose, A. (1991b) Development 113, 1193–1206.
33. Ward, T. A., Nael, A., Reeve, A. E., and Eccles, M. R. (1994) Cell Growth Diff. 5, 1015–1021.
34. Chalepakis, G., Wijholdhs, J., and Gruss, P. (1994b) Nucleic Acids Res. 22, 3131–3137.
35. Waters, S. H., Saik, K. U., and Stavnezer, J. (1989) Mol. Cell. Biol. 9, 5594–5601.
36. Williams, M., and Maizels, N. (1991) Genes & Dev. 5, 235323 61.
37. Read, A. P., and van Heyningen, V. (1994) J. Immunol. 148, 2909–2917.
38. Xu, L., Kim, M. G., and Marcu, K. B. (1992) Int. Immunol. 4, 875–887.
39. Walter, J., Dever, C. A., and Biggin, M. D. (1994) Genes & Dev. 8, 1678–1692.
40. Goward, P. A., and White, R. A. H. (1992) Development 116, 1163–1174.
41. Strutt, D. I., and White, R. A. H. (1994) Mol. Cell. Dev. 45, 195–200.
42. Bigler, J., and Eisenman, R. N. (1994) Mol. Cell. Biol. 14, 7621–7632.
43. Grab, Y., Aragoti, D., Laurer, P., Garzino, V., Charnot, D., Berenger, H., and Pradel, J. (1992) EMBO J. 11, 3375–3384.
44. Tomatsu, T., Shoji, H., Wakamatsu, Y., Kondoh, H., and Takahashi, N. (1993) Nature 365, 69–72.
45. Orlando, V., and Paro, R. (1993) Cell 75, 1187–1198.
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