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Pax-8, a Paired Domain-Containing Protein, Binds to a Sequence Overlapping the Recognition Site of a Homeodomain and Activates Transcription from Two Thyroid-Specific Promoters

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The *Pax-8* gene, a member of the murine family of paired box-containing genes (*Pax* genes), is expressed in adult thyroid and in cultured thyroid cell lines. The Pax-8 protein binds, through its paired domain, to the promoters of thyroglobulin and thyroperoxidase, genes that are exclusively expressed in the thyroid. In both promoters, the binding site of Pax-8 overlaps with that of TTF-1, a homeodomain-containing protein involved in the activation of thyroid-specific transcription. Pax-8 activates transcription from cotransfected thyroperoxidase and thyroglobulin promoters, indicating that it may be involved in the establishment, control, or maintenance of the thyroid-differentiated phenotype. Thus, the promoters of thyroglobulin and thyroperoxidase represent the first identified natural targets for transcriptional activation by a paired domain-containing protein.

The identification of genes controlling embryonal development of the fruit fly *Drosophila melanogaster* has had a profound impact in the study of development in vertebrates, since many of such genes contain highly conserved sequences that have been used to isolate the corresponding genes from other species. The vertebrate *HOX* gene family, for example, has been isolated by using the homeobox, a 180-bp-long DNA sequence present in genes from all eukaryotes studied thus far (1, 39). The homeobox encodes the homeodomain, which contains a protein motif structurally analogous to the helix-turn-helix motif found in prokaryotic transcriptional regulators (32, 38, 44) and is capable of sequence-specific DNA recognition (25). The murine *Pax* gene family has been isolated in an analogous manner (31) by using as a probe the paired box, a segment of DNA first identified in the *Drosophila* paired (*Prd*) gene (21) and subsequently shown to be present in several other *Drosophila* genes (9). On the basis of their sequences, the paired domains were originally grouped into three classes, denominated paired, pox meso, and pox neuro, from the names of the prototype *Drosophila* genes (10, 11). Recently, after the isolation of additional paired box-containing genes, a new grouping into six classes (I to VI) has been proposed (43). Like the homeobox, the paired box encodes a protein domain that is capable of specific DNA binding. The first binding site for a paired domain was identified, by using the Prd protein, in the promoter of the *Drosophila* even-skipped gene (42). Subsequently, derivatives of the even-skipped sequence have been used to demonstrate the DNA-binding activity of the vertebrate Pax-1 (12) and Pax-3 (27) proteins. Interestingly, some of the paired box genes also contain a homeobox, and it has been suggested that the combination of the two DNA-binding domains in the Prd protein confers a novel DNA-binding specificity, different from that displayed by either domain (42). Studies on the ectopic expression of the wild type and deletion mutations of *Prd* also indicate that both the homeodomain and the paired domain are needed for proper function of the protein (35). While the ability of the paired domain to bind specific DNA sequences suggests that the proteins containing such a structure may be involved in transcriptional control, the evidence supporting this notion is rather weak, since neither Prd nor Pax-3 can activate transcription from promoters containing the e5 sequence. In addition, Prd mutants do not alter the expression of the even-skipped gene. *Pax-1*, instead, is capable of activating transcription, but only from an artificial promoter containing multimerized binding sites. A *Pax-1* mutant (undulated), which has a single Gly-to-Ser replacement in its paired domain (7), shows an altered DNA-binding and transcriptional activity (12, 42). Undulated mice are characterized by abnormal development of the vertebral column and sternum (28), two sites of expression of *Pax-1* (16). This observation is indicative of an important developmental role for the DNA-binding and transcriptional activating properties of Pax-1 and, by analogy, perhaps of other Pax proteins. However, to date no natural promoter has been identified as a target for any of the paired domain-containing proteins.

The *Pax-8* gene encodes a protein which contains a paired domain but no homeodomain, and it is expressed in the developing central nervous system, kidney, and thyroid (37). We have been using the thyroid as a model to study cell-type-specific gene expression. The promoters of thyroglobulin (Tg) and thyroperoxidase (TPO), two genes exclusively expressed in the thyroid, contain several binding sites for TTF-1 (14, 18). TTF-1 is a homeodomain-containing protein which shows a restricted tissue distribution (29) and

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that is expressed from the onset of thyroid differentiation (34). Previous data suggested that TTF-1 could be the main regulator of the Tg promoter (14). While TTF-1 is also involved in regulating the TPO promoter, another sequence-specific DNA-binding protein, present in crude extracts of differentiated thyroid cells, has been implicated in controlling the thyroid-specific expression of the TPO gene (18).

We demonstrate in this paper that Pax-8 binds to the Tg and TPO promoters. In both cases, binding of Pax-8 occurs at a site overlapping the TATA box-proximal TTF-1-binding site. When introduced in nonthyroid cells, Pax-8 can activate transcription from cotransfected TPO and, albeit to a lesser extent, Tg promoters. These results suggest a role for *Pax-8* in thyroid-specific gene expression and identify a natural target for this paired domain-containing protein.

MATERIALS AND METHODS

Plasmids. P8IIBS was obtained by inserting a *Sal*I-*Xba*I fragment containing the full coding sequence of Pax-8 into the corresponding sites of Bluescript. BS-PB was generated by cloning the Pax-8 paired domain, amplified by the polymerase chain reaction, in the *Bam*HI-*Hin*dIII sites of Bluescript. Plasmid C27BXX22/1 contains the *Xho*I-*Pst*I fragment of Pax-8 cDNA cloned in Bluescript and was used as a hybridization probe. The expression vector used in transactivation experiments was generated by inserting the *Sal*I-*Xba*I fragment of P8IIBS in the corresponding sites of the pCMV5 vector (5). The Tg promoter linked to a chloramphenicol acetyltransferase reporter gene (Tg-WT) and the TPO promoters (TPO-WT, TPO-PM, and TPO-EM) linked to the luciferase reporter gene have been previously described (18, 40). pGEXPrdPB and pGEXPax-8PB were a gift of Claude Desplan.

RNA extraction and Northern analysis. Total RNA was prepared by the acid guanidinium thiocyanate phenol procedure, and poly(A)$^+$ RNA was prepared from this by oligo(dT) cellulose chromatography.

Thirty micrograms of total RNA and 4 μg of poly(A)$^+$ RNA were electrophoresed through a 1% agarose gel containing 3.7% formaldehyde and 20 mM morpholinepropanesulfonic acid (MOPS) buffer. RNA was then blotted onto nylon membranes (Hybond-N) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and hybridized overnight by the procedure of Church and Gilbert (13) at 65°C with either a riboprobe generated from plasmid C27BXX22/1 transcribing the *Xho*I-*Pst*I fragment with T7 polymerase or a DNA probe obtained by labelling the same fragment by random oligonucleotide priming.

The membranes were washed twice in 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 min.

Protein preparation. Nuclear extracts from FRTL-5 cells were prepared as previously described (14). Purified TTF-1 homeodomain was obtained from a bacterial expression system (15). pGEXPax-8PB and pGEXPrdPB fusion proteins were prepared as previously described (41).

For in vitro transcription and translation, plasmids P8IIBS and BS-PB containing Pax-8 cDNA and the Pax-8 paired domain, respectively, and a plasmid containing TTF-1 cDNA cloned in Bluescript (29) were linearized by using restriction sites at the 3′ end of the Bluescript polylinker. The sense RNA strand was synthesized with T3 or T7 RNA polymerase (Promega), according to the cloning direction. One-thirtieth of the RNA template was translated in a rabbit reticulocyte lysate by following the manufacturer's specifications (Promega), and an aliquot was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) to check the amounts of the protein.

Band shift assays, orthophenanthroline footprinting, methylation interference analysis, and DNase I footprinting. For gel shift assays, binding reactions were carried out in a buffer containing 20 mM Tris-HCl (pH 8.0), 75 mM KCl, 1 mM dithiothreitol, 1 mg of bovine serum albumin per ml, 1 to 3 mg of poly(dI-dC) per ml, and 10% glycerol. After 30 min of incubation at room temperature, free DNA and DNA-protein complexes were resolved on a 5% polyacrylamide gel run in 0.5× Tris-borate-EDTA for 2 to 3 h at 4°C. The gel was dried and then exposed to an X-ray film at −80°C overnight. In competition experiments, increasing molar excesses of competitor were added to a standard binding reaction mixture prior to the addition of the labelled substrate and incubated for 10 min at room temperature. In situ orthophenanthroline-copper (Cu^{2+}) footprinting was carried out essentially as described by Kuwabara and Sigman (33). The *Spe*I-*Nhe*I fragment (5′ end labelled) and TTF-1 or Pax-8 in vitro-translated protein were used for a gel shift assay carried out as described above. After running, the polyacrylamide gel was immersed in 200 ml of Tris-HCl, pH 8.0. Then 20 ml of a solution containing 2 mM orthophenanthroline–0.45 mM $CuSO_4$ was added together with 20 ml of a solution of 58 mM 3-mercaptopropionic acid, and the digestion was allowed to proceed at room temperature for 15 min. To quench the reaction, 20 ml of 28 mM 2,9-dimethylorthophenanthroline was added, and the resulting solution was allowed to stand for 2 min. The gel was then rinsed extensively with distilled water and exposed to X-ray film for 30 min at 4°C. The bands of interest were cut from the gel and eluted overnight at room temperature in 0.1% SDS–0.5 M ammonium acetate–10 mM magnesium acetate. Eluted DNA was precipitated and resuspended in distilled water. An equal number of counts of each sample were dried, resuspended in 80% formamide–10 mM NaOH–1 mM EDTA–0.1% bromophenol blue–0.1% xylene cyanol, and loaded on a 10% sequencing gel. The gel was then exposed to X-ray film at −80°C overnight.

For methylation interference analysis and DNase I footprinting assays, experiments were performed as previously described (18).

Cell culture and transfection. HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. For transient expression assays cells were plated at 3×10^5 cells per 60-mm-diameter tissue culture dish 5 to 8 h prior to transfection. Transfections were carried out by the calcium phosphate coprecipitation technique, and cells were exposed to the precipitate for 12 to 14 h. One microgram of a plasmid containing the β-galactosidase cistron under the control of the cytomegalovirus enhancer/promoter was used to monitor for transfection efficiency. Cell extracts were prepared 48 h after transfection and the chloramphenicol acetyltransferase, luciferase, and β-galactosidase activities were determined as previously described (17, 26, 30).

RESULTS

Expression of Pax-8 mRNA in rat tissues and cell lines. Previous in situ hybridization studies demonstrated that Pax-8 mRNA is present in developing brain, kidney, and thyroid and in adult kidney tissue. In the developing thyroid, *Pax-8* is expressed, like *TTF-1* (34), at the onset of organogenesis in the endodermal cells of the budding thyroid anlage (37) and its expression is maintained until day 16.5 p.c. To test whether *Pax-8* is also expressed in adult thyroid, an *Xho*I-*Pst*I restriction fragment, corresponding to nucleotides

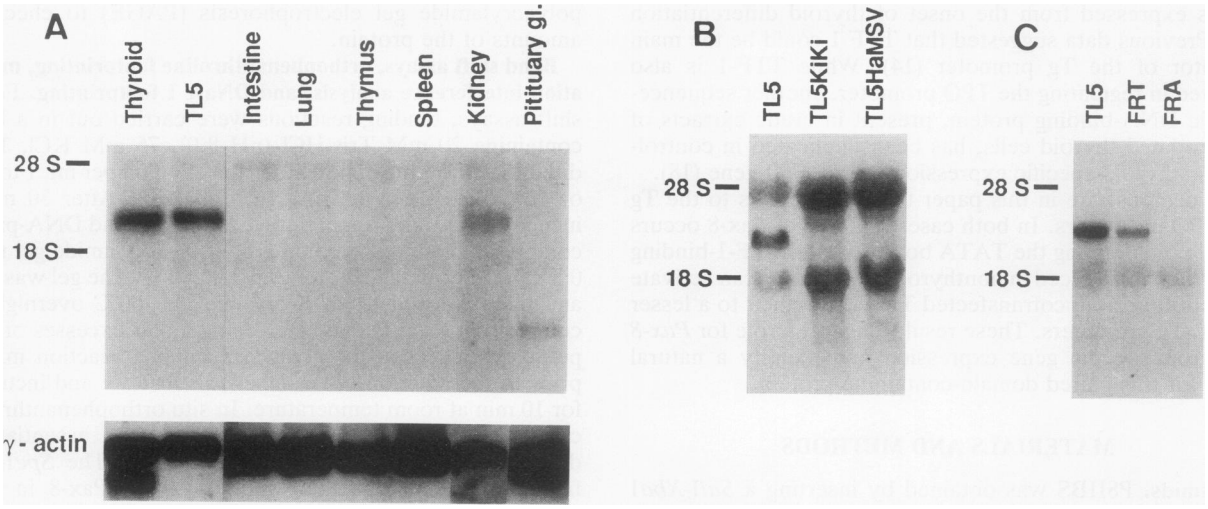

FIG. 1. Northern blot analysis of Pax-8 transcripts in rat adult tissues and in rat thyroid cell lines. (A) Four micrograms of poly(A)$^+$ RNAs from several mouse adult tissues was hybridized with a Pax-8-specific riboprobe. A transcript of 3.1 kb is detected in FRTL-5 cells (TL5), in thyroid, and in kidney. A smaller transcript is detected in the pituitary gland. (B) Thirty micrograms of total RNA from wild-type FRTL-5 cells (TL5) and FRTL-5 cells transformed with K-*ras* (TL5KiKi) or H-*ras* (TL5HaMSV) were hybridized to the same riboprobe as in panel A. Cross hybridization of the riboprobe with the large amount of rRNA loaded on this gel is clearly visible. (C) Total RNA (30 μg) from FRT, FRA, and FRTL-5 (TL5) cells were hybridized to a DNA probe corresponding to the same region of Pax-8 used in panels A and B.

855 to 1156 of the cloned Pax-8 cDNA, was hybridized to a panel of RNAs obtained from adult rat tissues and to RNA extracted from FRTL-5 cells, a cultured cell line that displays most of the differentiated phenotype of thyroid follicular cells (3). This experiment (Fig. 1A) revealed the presence of a 3.1-kb Pax-8 mRNA in both adult thyroid tissue and FRTL-5 cells. An mRNA of the same size was also observed in kidney, as reported previously for the mouse (37). Interestingly, we detected the presence of a smaller mRNA in the anterior pituitary which was not detected by in situ hybridization (37), probably because of the use of different fragments of the Pax-8 cDNA as hybridization probes. The nature of the pituitary RNA was not investigated further.

The *Xho*I-*Pst*I Pax-8 probe described above was also hybridized to RNA extracted from a panel of dedifferentiated thyroid cell lines. The TL5KiKi (24) and TL5HaMSV (22) cell lines (Fig. 1B) are FRTL-5 derivatives obtained by transformation with the v-Ki-*ras* or v-Ha-*ras* oncogenes, respectively, while the FRA cell line (Fig. 1C) was obtained from a rat thyroid tumor (4). The absence of Pax-8 mRNA in these cells correlates well with the absence of differentiation. In the FRT cell line (2), Pax-8 mRNA is present at a concentration comparable to that observed in FRTL-5 cells (Fig. 1C). FRT cells were obtained from normal thyroid tissue. Their epithelial appearance and capacity to induce carcinomas after transformation suggested that they could have been derived from thyroid follicular cells (23). However, the absence of all known markers of thyroid differentiation made it difficult to be sure about their origin. The presence of Pax-8 mRNA strongly supports a relationship between FRT and thyroid follicular cells.

Pax-8 binds to the Tg promoter to a site which overlaps the binding site of TTF-1. The restricted expression of *Pax-8* in developing and adult thyroid, together with the recently demonstrated DNA-binding activity (27, 42) and transcriptional activation properties (12) of some paired domain-containing proteins, was highly suggestive of a role for *Pax-8* in controlling thyroid-specific transcription. The promoter of Tg, which is specifically expressed in differentiated thyroid cells in culture (36), was used to test this hypothesis. A pseudo-wild-type Tg promoter (Fig. 2A), where two unique restriction sites have been inserted by site-directed mutagenesis (40), was cut into three similarly sized fragments, and each of the fragments was tested for the binding to in vitro-produced Pax-8 protein by gel shift assay (20). Pax-8 is able to form a complex with two Tg promoter fragments (Fig. 2B), although the complex formed with the *Nhe*I-*Spe*I fragment is by far the most abundant. A complex of similar mobility is observed with crude nuclear extract from the FRTL-5 cell line, suggesting that an active form of Pax-8 is present in this extract. It was then decided to concentrate on the complex formed with the *Nhe*I-*Spe*I fragment in order to define the Pax-8-binding site better, and the weak interaction with the *Sal*I-*Spe*I fragment was not characterized further.

To map the sequence recognized by Pax-8 and to determine its position relative to that of TTF-1, which has a high-affinity binding site within the same *Nhe*I-*Spe*I fragment (14), a scaled-up band shift experiment was performed (Fig. 2C) using in vitro-synthesized Pax-8 and TTF-1. The entire gel was treated with phenanthroline, and the free DNA and the DNA bound by Pax-8 or TTF-1 were eluted from the gel and analyzed on a sequencing gel. Surprisingly, the protection obtained with the two proteins overlap, the TTF-1 protection being completely included within the sequence protected by Pax-8 (Fig. 2D and E). These results suggested that Pax-8 should be able to form a complex with the C oligonucleotide (Fig. 2E) that we have previously used to assay and purify TTF-1 (14). The mobility shift assay shown in Fig. 3 demonstrates that both TTF-1 and Pax-8 are able to bind the C oligonucleotide, resulting in complexes with identical electrophoretic mobilities. This experiment also provides an explanation as to why only TTF-1 was detected in our previous binding assays with the C oligonucleotide. Previous band shift assays were performed at a KCl concentration of 200 mM (14). It is clear from Fig. 3,

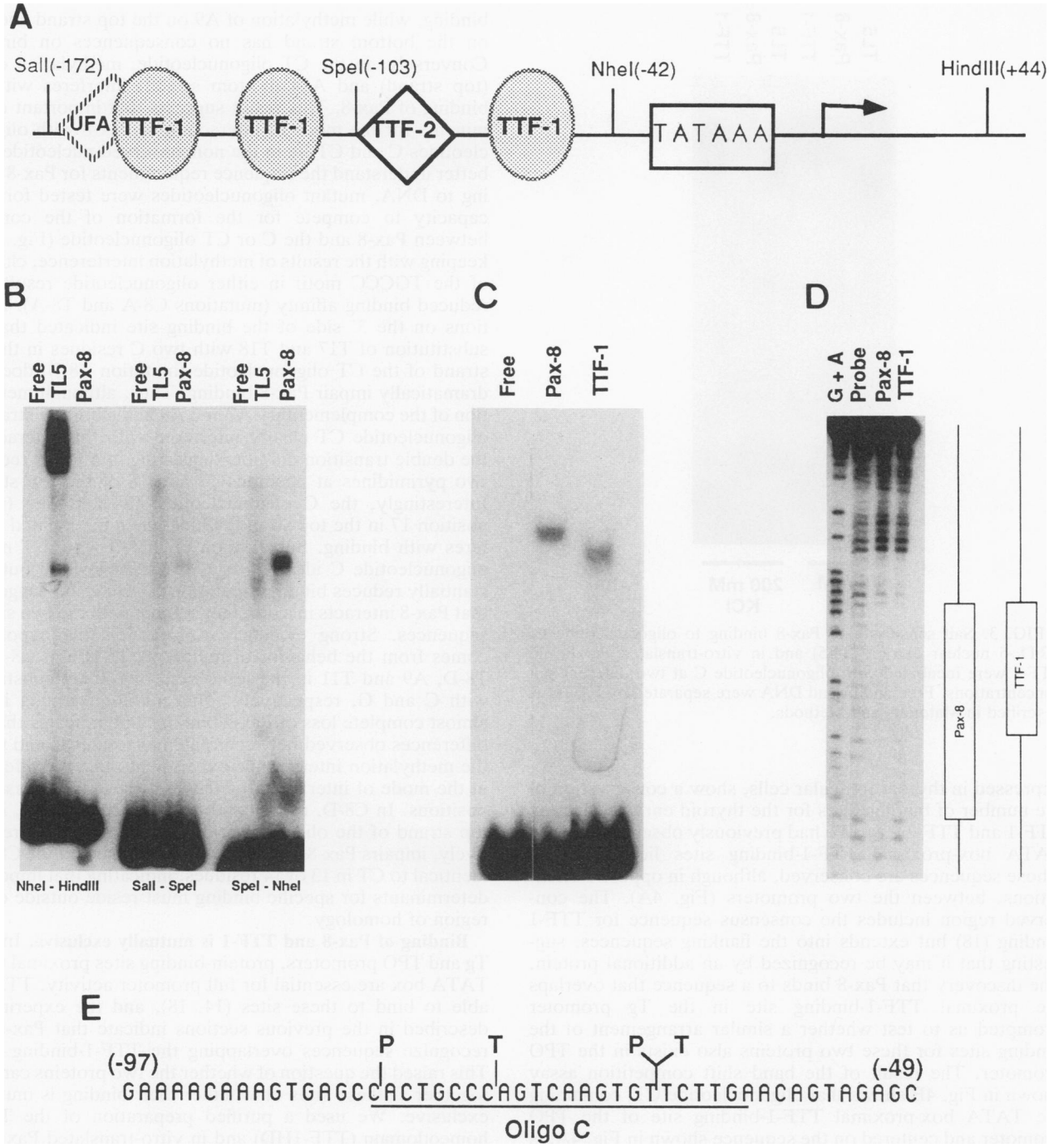

FIG. 2. A binding site for Pax-8 is present in the Tg promoter. (A) Schematic representation of the interaction of nuclear factors with the minimal Tg promoter. TTF-1 and TTF-2 are thyroid-specific factors, while UFA is an ubiquitous factor. (B) The Tg promoter was divided into three fragments by restriction digestions, and the resulting fragments were then added in a binding assay to a nuclear extract from FRTL-5 (TL5) cells and to Pax-8 protein obtained by in vitro translation. (C) A scaled-up band shift performed on the *SpeI-NheI* fragment with in vitro-translated TTF-1 and Pax-8. Orthophenanthroline footprints of the free and bound DNA, obtained from the gel shown in panel D, were analyzed on a denaturing gel; the region protected by Pax-8 and TTF-1 is shown on the side of the autoradiogram. (E) Summary of the footprinting results. P and T indicate the borders of the protection obtained with Pax-8 and TTF-1, respectively. The sequence of oligonucleotide C which contains both binding sites is also indicated.

however, that Pax-8 binds poorly at this salt concentration, while it forms a readily detectable complex at 75 mM KCl. It follows from this experiment that binding assays performed with this oligonucleotide in crude extracts from FRTL-5 cells at a low salt concentration are likely to have detected a mixture of TTF-1 and Pax-8 (6).

Pax-8 and TTF-1 recognize overlapping sequences in the TPO promoter. The Tg and TPO promoters, both exclusively

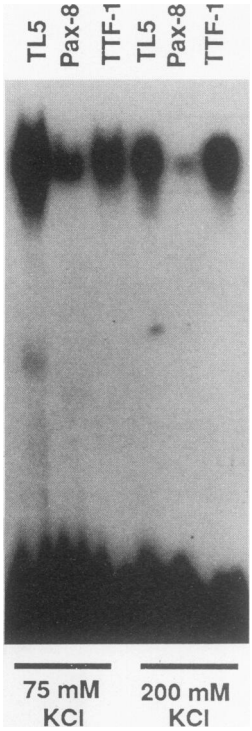

FIG. 3. Salt sensitivity of Pax-8 binding to oligonucleotide C. FRTL-5 nuclear extract (TL5) and in vitro-translated Pax-8 and TTF-1 were incubated with oligonucleotide C at two different salt concentrations. Free and bound DNA were separated by PAGE as described in Materials and Methods.

expressed in thyroid follicular cells, show a conservation of the number of binding sites for the thyroid enriched factors TTF-1 and TTF-2 (18). We had previously observed that the TATA box-proximal TTF-1-binding sites lie in regions whose sequences are conserved, although in opposite orientations, between the two promoters (Fig. 4A). The conserved region includes the consensus sequence for TTF-1 binding (18) but extends into the flanking sequences, suggesting that it may be recognized by an additional protein. The discovery that Pax-8 binds to a sequence that overlaps the proximal TTF-1-binding site in the Tg promoter prompted us to test whether a similar arrangement of the binding sites for these two proteins also exists in the TPO promoter. The result of the band shift competition assay shown in Fig. 4B shows that oligonucleotide CT, containing the TATA box-proximal TTF-1-binding site of the TPO promoter and centered on the sequence shown in Fig. 4A, is bound by Pax-8 and, furthermore, that it has an higher affinity for Pax-8 than oligonucleotide C does. Thus, both Pax-8 and TTF-1 recognize oligonucleotides containing the sequence conserved between the Tg and TPO promoters. To define the binding site of Pax-8 better, the contacts with purines on both strands of the C (Tg) and CT (TPO) oligonucleotides were determined by using a methylation interference assay (Fig. 4C). The results of this experiment are summarized in Fig. 4D. Pax-8 recognizes the same residues within the shared TGCCC motif, but despite the extensive sequence homology between the two oligonucleotides used, the pattern of interference changes within the common, adjacent AG/CTC sequence. In the C oligonucleotide, methylation of G10 on the top strand interferes with binding, while methylation of A9 on the top strand and A14 on the bottom strand has no consequences on binding. Conversely, in the CT oligonucleotide, methylation of A9 (top strand) and A14 (bottom strand) interferes with the binding of Pax-8. This result suggests that important determinants for the mode of interaction of Pax-8 with oligonucleotides C and CT lie in the nonconserved nucleotides. To better understand the sequence requirements for Pax-8 binding to DNA, mutant oligonucleotides were tested for their capacity to compete for the formation of the complex between Pax-8 and the C or CT oligonucleotide (Fig. 5). In keeping with the results of methylation interference, changes of the TGCCC motif in either oligonucleotide resulted in reduced binding affinity (mutations C8-A and T8-A). Mutations on the 3′ side of the binding site indicated that the substitution of T17 and T18 with two C residues in the top strand of the CT oligonucleotide (mutation T8-B) does not dramatically impair Pax-8 binding. Thus, although methylation of the complementary A7 and A8 in the bottom strand of oligonucleotide CT clearly interfered with this interaction, the double transition did not, suggesting that Pax-8 requires two pyrimidines at positions 17 and 18 of the top strand. Interestingly, the C oligonucleotide has a purine (G) at position 17 in the top strand, which when methylated interferes with binding. Substitution of this G with a T makes oligonucleotide C identical to CT at this position but substantially reduces binding (mutation C8-B), again suggesting that Pax-8 interacts in a different manner with the two similar sequences. Strong evidence in favor of this hypothesis comes from the behavior of mutations T8-D and C8-D. In T8-D, A9 and T11 in the top strand of CT are substituted with C and G, respectively. This mutation results in the almost complete loss of Pax-8 binding and indicates that the differences observed between oligonucleotides C and CT in the methylation interference experiment reflect a difference in the mode of interaction of Pax-8 at these two conserved positions. In C8-D, the substitution of G10 and G17, in the top strand of the oligonucleotide C, with C and T, respectively, impairs Pax-8 binding, even if oligonucleotide C8-D is identical to CT in 13 of 14 residues, indicating that important determinants for specific binding must reside outside of the region of homology.

Binding of Pax-8 and TTF-1 is mutually exclusive. In both Tg and TPO promoters, protein-binding sites proximal to the TATA box are essential for full promoter activity. TTF-1 is able to bind to these sites (14, 18), and the experiments described in the previous sections indicate that Pax-8 can recognize sequences overlapping the TTF-1-binding sites. This raised the question of whether the two proteins can bind together at these sites or whether their binding is mutually exclusive. We used a purified preparation of the TTF-1 homeodomain (TTF-1HD) and in vitro-translated Pax-8, so that the complexes formed by either protein could be clearly distinguished. When increasing quantities of Pax-8 are added, together with a saturating concentration of TTF-1HD, to a binding reaction mixture containing the C oligonucleotide, only small amounts of Pax-8–C complex appear, while most of the probe remains occupied by TTF-1HD (Fig. 6A). We conclude that, at least in vitro, the two proteins cannot bind together to the same DNA molecule, since even if most of the probe is complexed with proteins, no ternary complex is observed. Similar results are observed with the CT oligonucleotide (Fig. 6B).

The binding sites of Pax-8 in the Tg and TPO promoters define a new class of binding sites for paired domains. A sequence contained in the *Drosophila* even-skipped pro-

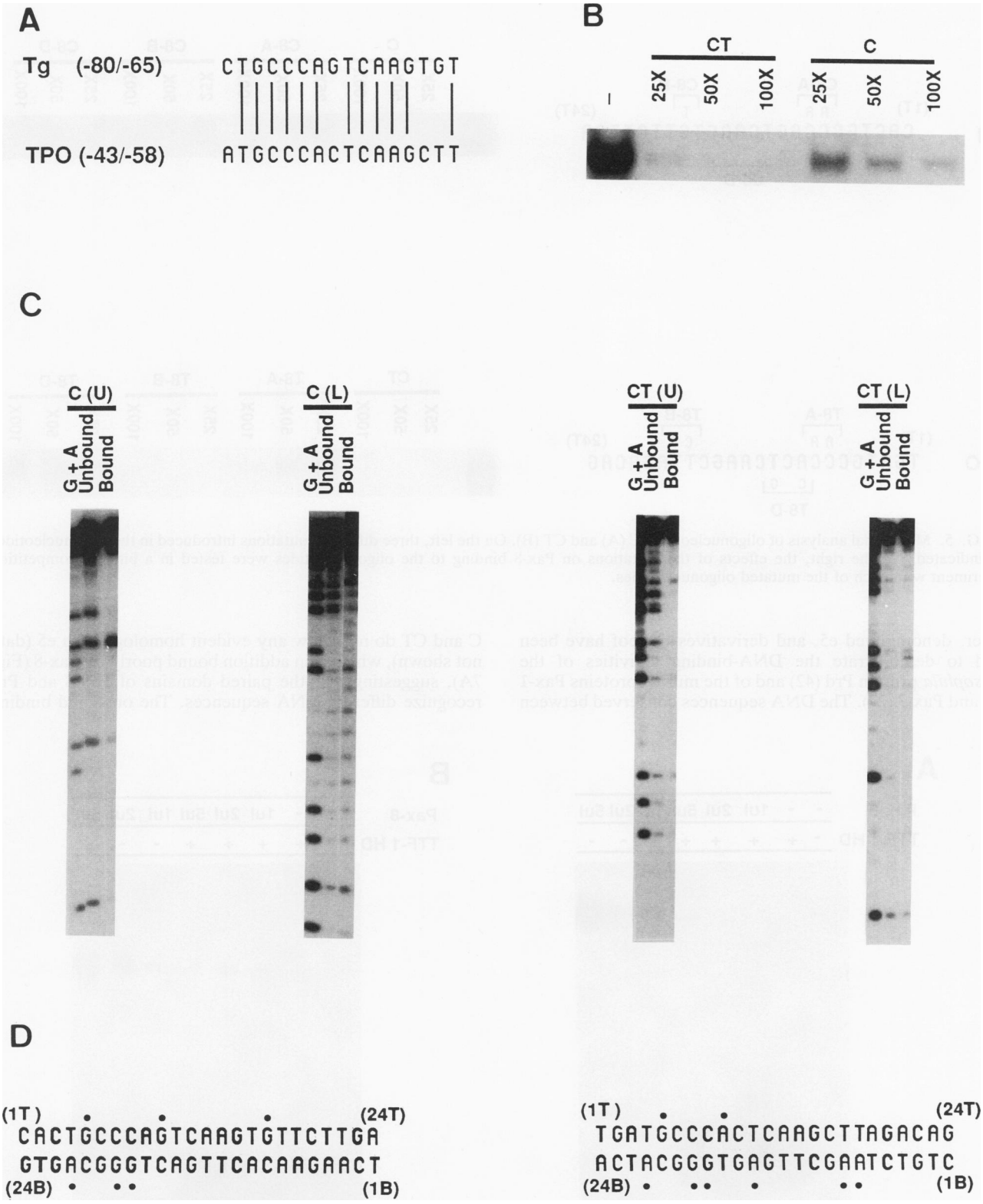

FIG. 4. Comparison of the Pax-8-binding sites in the promoters of Tg and TPO. (A) Sequence homology between the Pax-8-binding sites in the Tg and TPO promoters; numbers in parentheses indicate the position of the sequence in each promoter, relative to the major transcription start site. (B) Pax-8 binding to oligonucleotide CT in the absence (−) or in the presence of the indicated amounts of C and CT competitor oligonucleotides. (C) Methylation interference analysis of Pax-8 on oligonucleotides C and CT. Residues that interfere with Pax-8 binding when methylated are indicated by dots and were determined as described in Materials and Methods. (D) Summary of Pax-8 contacts on the two sequences analyzed.

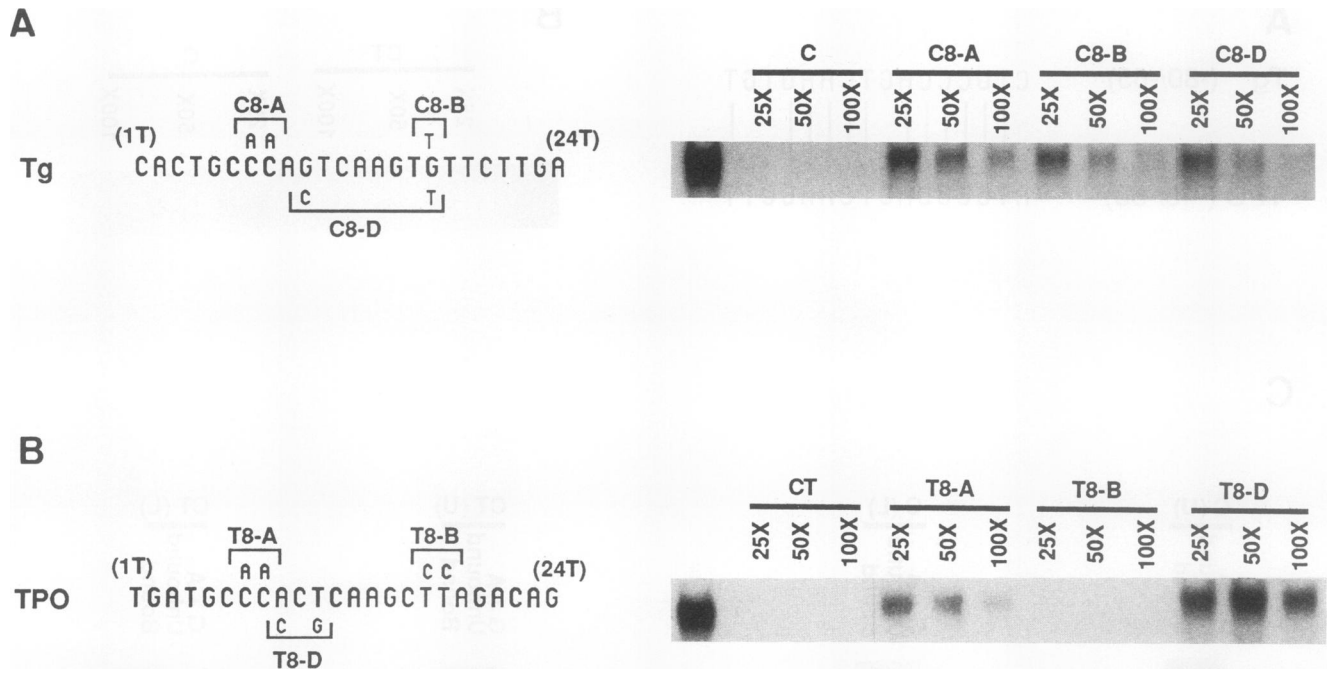

FIG. 5. Mutational analysis of oligonucleotides C (A) and CT (B). On the left, three different mutations introduced in the oligonucleotides are indicated. On the right, the effects of the mutations on Pax-8 binding to the oligonucleotides were tested in a binding competition experiment with each of the mutated oligonucleotides.

moter, denominated e5, and derivatives thereof have been used to demonstrate the DNA-binding activities of the *Drosophila* protein Prd (42) and of the murine proteins Pax-1 (12) and Pax-3 (27). The DNA sequences conserved between C and CT do not show any evident homology with e5 (data not shown), which is in addition bound poorly by Pax-8 (Fig. 7A), suggesting that the paired domains of Pax-8 and Prd recognize different DNA sequences. The observed binding

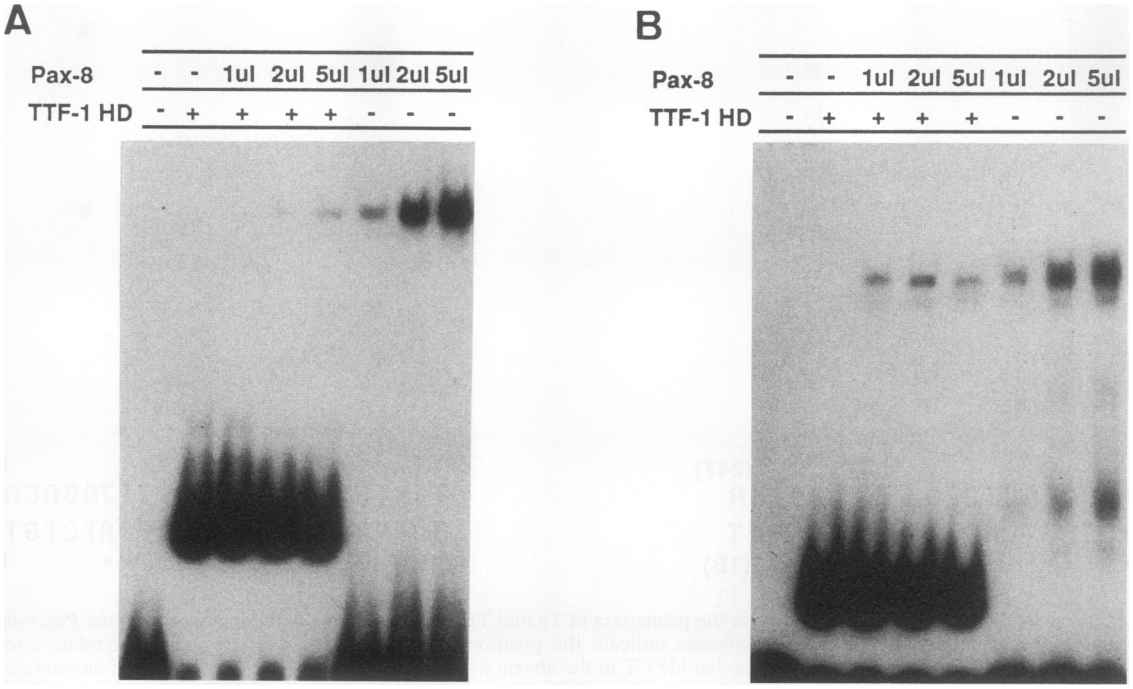

FIG. 6. Pax-8 and TTF-1 bind to both the oligonucleotide C and CT sequences in a mutually exclusive fashion. Increasing amounts of in vitro-translated Pax-8 were incubated, alone or together with a fixed amount of TTF-1 homeodomain produced in bacteria, in a binding assay with either oligonucleotide C (A) or oligonucleotide CT (B).

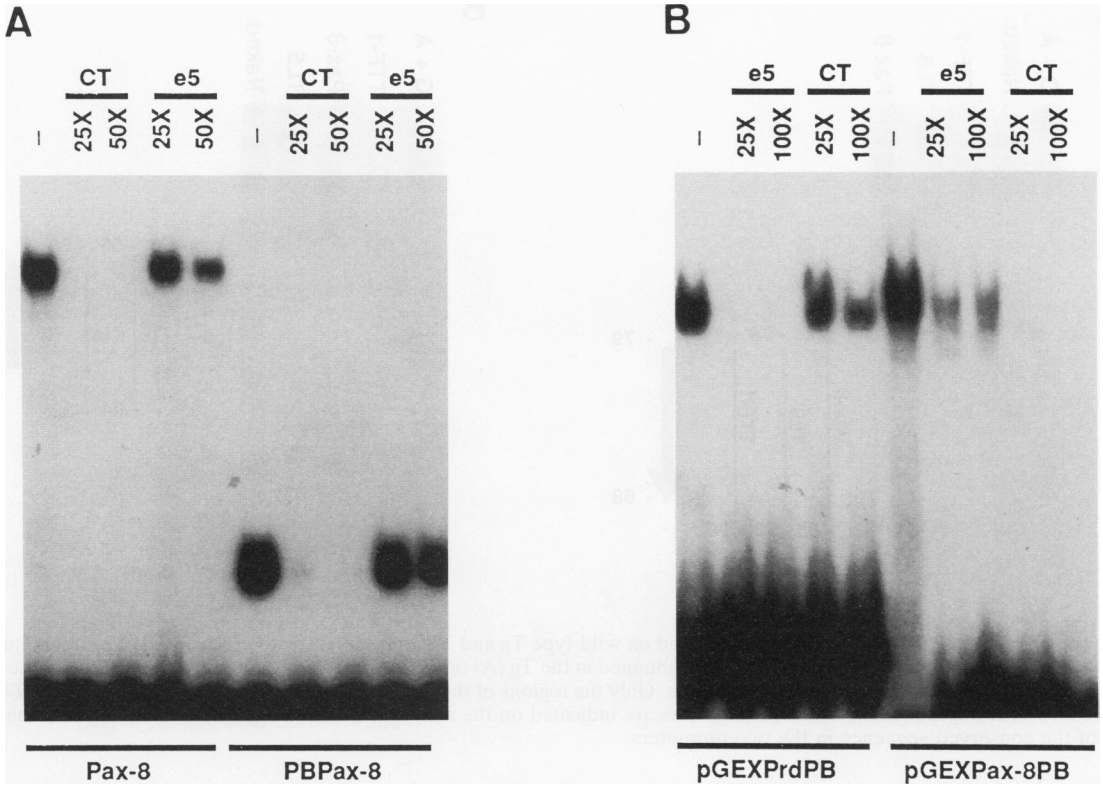

FIG. 7. DNA-binding specificities of the paired domains of Prd and Pax-8. (A) In vitro-translated, full-size Pax-8 or the Pax-8 paired domain (PBPax-8) was incubated with labelled oligonucleotide CT in the absence of competitor (−) or in the presence of the indicated molar excesses of oligonucleotide CT or e5. (B) Sequence-specific DNA binding of Prd and Pax-8. Prd (pGEXPrdPB) and Pax-8 (pGEXPax-8PB) paired domains, produced as glutathione *S*-transferase fusion proteins in bacteria, were incubated in a binding assay with oligonucleotides e5 and CT, respectively, in the absence (−) or in the presence of the indicated molar excesses of oligonucleotides e5 and CT as competitors. Free and bound DNA were separated by PAGE as described in Materials and Methods.

specificity of Pax-8 is a property of its paired domain alone, since a deletion protein that retains only amino acids 1 to 128 of Pax-8 (PBPax-8) is still able to show a strong preference for CT versus e5 (Fig. 7A). In addition, this deletion protein binds DNA with an affinity consistently higher than that of the full-size protein, suggesting that the carboxy terminus of Pax-8 may exert a negative influence on the binding activity of the paired domain.

Fusion proteins containing the paired domain of either Pax-8 (pGEXPax-8PB) or Prd (pGEXPrdPB) fused to glutathione *S*-transferase were purified from overproducing *Escherichia coli* strains and challenged with oligonucleotides e5 and CT. The results of such an experiment (Fig. 7B) clearly show that while the paired domain of Prd binds only to e5, that of Pax-8 shows a strong preference for CT, indicating that the paired domains of these two proteins have quite different DNA-binding specificities.

Pax-8 is a transcriptional activator. The ability of Pax-8 to bind to a sequence that overlaps the TATA-proximal TTF-1-binding site in both Tg and TPO promoters prompted us to reinvestigate the nature of the protein(s) binding to these sites in crude FRTL-5 extracts. To this end, footprints obtained in these regions with crude FRTL-5 extracts were compared with those obtained with either TTF-1HD or pGEXPax-8PB. In the Tg promoter, the region protected by the crude extract coincides, as shown previously (29), with the TTF-1 footprint and does not extend into the sequence exclusively protected by Pax-8 (Fig. 8A), indicating that this factor cannot bind to the Tg promoter when present at the concentration found in crude nuclear extracts, either because of its low affinity or because of the much higher affinity of TTF-1 for the same region. Conversely, in the corresponding region of the TPO promoter, the FRTL-5 extract gives a footprint, larger than that of TTF-1, which extends into the sequence specifically protected by Pax-8 (Fig. 8B). This evidence suggests that the FRTL-5 enriched factor, previously identified on the basis of a similar comparison between FRTL-5 and TTF-1 footprints (18), could be Pax-8. The differences observed between the 3′ borders of the FRTL-5 and Pax-8 footprints may be due to the different structure of the FRTL-5- and *E. coli*-produced Pax-8 or to the mutually exclusive binding of Pax-8 and TTF-1, which would result in a mixed population of DNA molecules bound by one or the other protein. To eliminate the interference of TTF-1 in such an analysis, we used a previously described TPO promoter mutant (EM) that abolishes the binding of TTF-1 to the TATA-proximal site but still shows the binding of the additional activity present in FRTL-5 extracts (18). On the promoter harboring the EM mutation, pGEXPax-8PB and FRTL-5 protect identical regions (Fig. 9, EM). Additionally, pGEXPax-8PB does not footprint the PM mutant of the TPO promoter (Fig. 9, PM). This mutation changes the TGCCC motif that we have proposed as an important sequence element for Pax-8 binding. The weak footprint obtained with FRTL-5 extracts is presumably due to the previously noted residual affinity of this mutant for TTF-1 (18). Taken to-

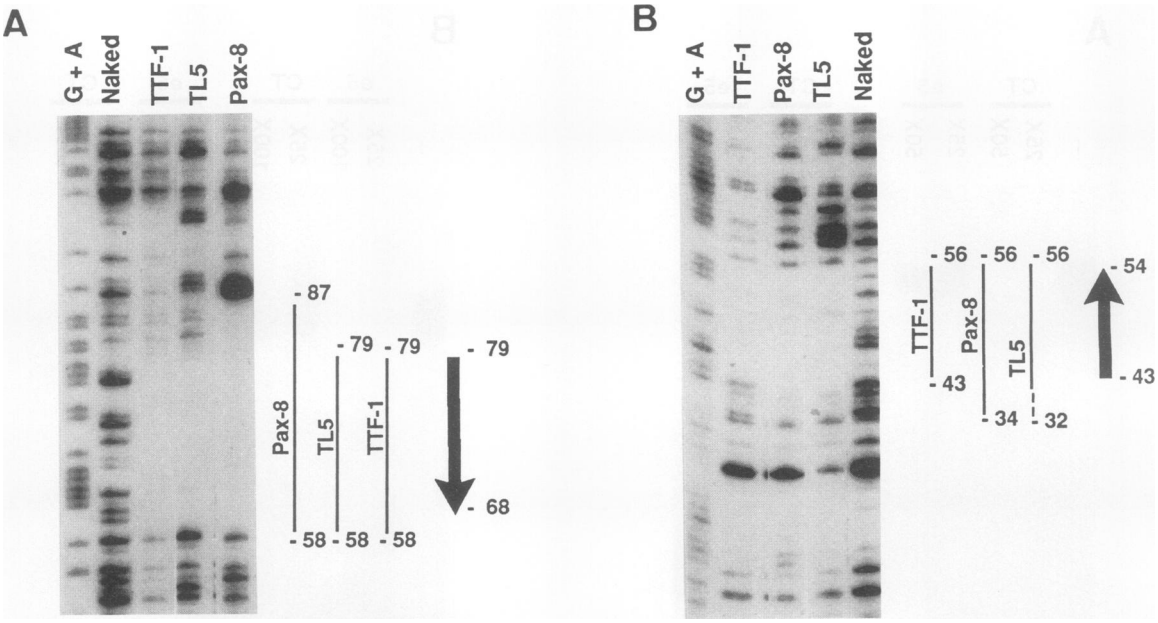

FIG. 8. Comparison of the DNase I footprints obtained on wild-type Tg and TPO promoters with crude FRTL-5 extracts, purified TTF-1 homeodomain, and Pax-8 paired domain. The footprints obtained in the Tg (A) or the TPO (B) promoter with crude FRTL-5 extracts, purified TTF-1 homeodomain, and Pax-8 paired domain are shown. Only the regions of the two promoters where the binding sites for TTF-1 and Pax-8 overlap are shown. The extents of the different footprints are indicated on the side of the autoradiogram. Arrows indicate the position and the polarity of the conserved sequence in the two promoters.

gether, these data strongly suggest that Pax-8, in crude FRTL-5 extracts, is able to footprint the TPO promoter and presumably corresponds to the previously observed novel thyroid-specific factor (18).

A role for the binding of Pax-8 to the Tg and TPO promoters was directly investigated by cotransfection of a Pax-8 expression vector with either Tg-chloramphenicol acetyltransferase (40) or TPO-luciferase (18) into HeLa cells, which do not contain any Pax-8 (data not shown). When transfected alone, both promoters show very low transcriptional activity in these cells (Fig. 10). Cotransfection of Pax-8 results in 20- to 50-fold stimulation of TPO transcription, while Tg is only stimulated 2- to 4-fold. Similar experiments carried out with TPO promoters containing the EM or PM mutation correlate well with the binding of Pax-8, since the EM promoter is stimulated as well as the wild type, while PM shows very little or no *trans*-activation, indicating that the observed activation is a direct consequence of the binding of Pax-8 to the TPO promoter.

DISCUSSION

DNA-binding properties of Pax-8. The binding sites for Pax-8 in the Tg and TPO promoters, characterized in this study, show some interesting properties. The Pax-8-binding sites extend for at least 15 nucleotides, as determined by phenanthroline footprinting, methylation interference, and the effects of mutations. This target size is larger than that observed for the homeodomain (25) and correlates with the size of the paired domain, which is roughly twice as large as the homeodomain. Since Pax-8 has been reported not to dimerize (12), the protection observed is most likely due to the binding of a monomer of the protein. Pax-8 binds to the promoters of Tg and TPO, in regions displaying extensive sequence homology. Interestingly, in both cases, the binding site of Pax-8 overlaps with that of TTF-1, a homeodomain-containing protein that has been implicated as an important factor in the control of thyroid-specific gene expression (14, 18). This conservation in two otherwise unrelated genes suggests that the overlapping arrangement of the two binding sites is under selective pressure and hence has a functional relevance. Methylation interference and mutational studies of the two binding sites for Pax-8 reveal that the conserved TGCCCAG/CT motif is important for binding but also suggest that the surrounding sequences dictate distinct modes of interaction with the conserved core. The Pax-8 binding site, found in the C and CT oligonucleotides, does not show any similarity with either the e5 or PRS-1 sequence, which is bound by Prd and Pax-3 or by Pax-1, respectively (12, 27, 42). We have demonstrated in this study that the paired domain of Prd does not bind to CT and, conversely, that of Pax-8 does not recognize e5. Taken together with the previously reported inability of Pax-1 to bind to e5 (12), three distinct binding specificities of paired domains can be defined by DNA sequences e5 (bound by Prd and Pax-3), PRS-1 (bound by Pax-1), and CT (bound by Pax-8). These distinct DNA-binding specificities correlate well with classifications of the paired domains on the basis of their sequences, since Prd and Pax-3, Pax-1, and Pax-8 belong to three distinct classes (10, 43). It should be easy to test whether all paired domains of each class share DNA-binding specificity.

Pax-8 and thyroid-specific transcription. We show in this paper that *Pax-8* expression is maintained in the adult thyroid and in cultured cell lines that display the differentiated phenotype of the thyroid follicular cells. The expression of *Pax-8* correlates well with thyroid differentiation, since several dedifferentiated thyroid cell lines do not have detectable Pax-8 mRNA. The only exception is represented by the FRT cell line, which was derived from adult thyroid tissue

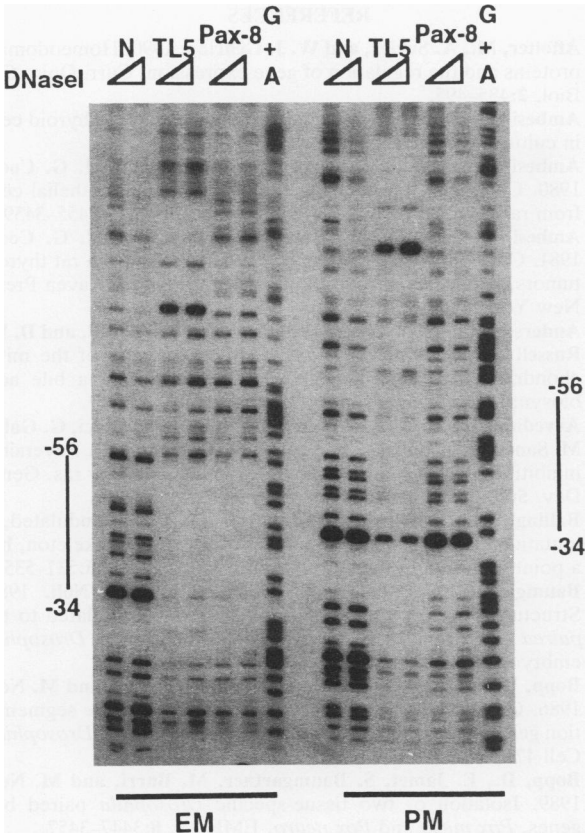

FIG. 9. DNase I footprints on TPO promoter mutants. Footprints obtained with mutants EM and PM with FRTL-5 nuclear extracts (TL5) or Pax-8 paired domain (Pax-8) are shown. N indicates the DNase I digestion products obtained in the absence of added proteins. All digestions were carried out at two different DNase I concentrations. The region from −56 to −34 is indicated for both mutants.

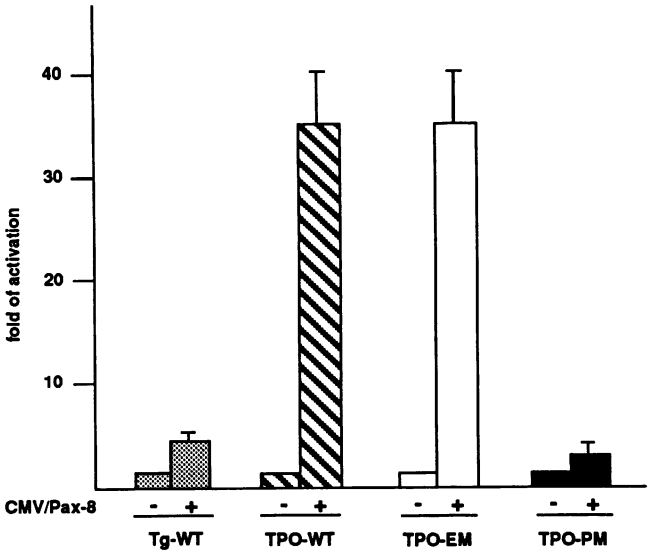

FIG. 10. Transactivation of the Tg and TPO promoters by Pax-8. HeLa cells were transiently cotransfected with 10 μg of the reporters Tg-WT, TPO-WT, TPO-EM, or TPO-PM and 0.2 μg of either the expression vector CMV/Pax8 (+) or the vector CMV5 (−), which does not contain any insert. The activation values were obtained by dividing the enzymatic activity present in extracts of cells transfected with CMV/Pax-8 by the activity obtained with CMV5.

and had not previously been found to display any of the known thyroid-specific markers. The presence of Pax-8 mRNA indicates that FRT cells are most likely derived from thyroid follicular cells and also suggests that the expression of Pax-8 is regulated independently from that of the other thyroid differentiation markers. This result also indicates that *Pax-8* expression is not sufficient to produce the differentiated phenotype of thyroid cells.

The presence of Pax-8 mRNA in the thyroid and its correlation with differentiation suggested that it could nevertheless play a role in the induction or maintenance of differentiation. This notion is further strengthened by the observation that Pax-8 can activate transcription from the promoters of Tg and TPO. In keeping with the higher affinity of Pax-8 for the sequence present in the CT oligonucleotide, the TPO promoter is activated much more efficiently than that of Tg. It should be noted that the Pax-8-binding sites in the two promoters are at different distances from the TATA box and in opposite orientations. It is conceivable that these differences play an important role in establishing the observed differential activation.

Transcriptional activation, at least in the TPO promoter, must be a direct effect of Pax-8 binding, since the PM mutation, which abolishes such interaction, also abolishes transcriptional activation by Pax-8 in HeLa cells. Previous transcription activation studies with other paired domain-containing proteins resulted in contradictory results. Pax-3 is unable to activate transcription from promoters containing the e5 sequence, but its carboxy terminus functions as a transcriptional activator when fused to the Gal4 DNA-binding domain (27). Furthermore, neither mutation (19) nor ectopic expression (35) of *Prd* influences even-skipped gene transcription, suggesting that the Prd protein does not control this promoter. Hence, either Prd and Pax-3 are not transcriptional activators or their effects are not exerted through the e5 sequence. Conversely, the Pax-1 protein is able to activate transcription from a promoter containing multimerized PRS-1, a sequence whose natural context, if any, is unknown (12). In the case of the TPO promoter, we have detected a single binding site for Pax-8 along the entire promoter sequence used in the *trans*-activation studies (data not shown), which indicates that the presence of multiple binding sites is not an essential requirement for efficient transcriptional activation by Pax-8. This is in contrast, for example, to TTF-1, for which several binding sites are found in both Tg (14) and TPO (18) promoters. This natural arrangement seems to reflect intrinsic properties of the protein, since in artificial promoter constructs a single binding site also cannot mediate *trans*-activation by TTF-1 (15a).

Interestingly, the Tg promoter also contains a TATA box-proximal binding site for Pax-8 but is not activated efficiently. We believe that it is unlikely that such a difference is due only to the lower affinity of Pax-8 for the Tg promoter, since even by increasing the amounts of the Pax-8 expression vector used in the cotransfection experiments, the Tg promoter is never activated more than two- to threefold (data not shown). It is more likely that Pax-8 has specific spatial requirements to function as activator of transcription, and a systematic comparison of Tg and TPO promoters may be instrumental in elucidating this property.

The behavior of the EM mutant supports a role for Pax-8

in the more physiological context of the differentiated thyroid cell line FRTL-5. We have previously reported that the EM mutation abolishes binding of TTF-1 at the TATA box-proximal site and yet shows a relatively high transcriptional activity in thyroid cells (40% of the wild-type promoter) which correlates with the persistence of the binding of a novel, thyroid-specific factor (18). Here we present data showing that the EM mutation does not interfere with either binding or transcriptional activation by Pax-8 in nonthyroid cells, suggesting that this protein could be responsible for the high residual activity of this mutant in thyroid cells and hence, at least in part, for the thyroid-specific expression of the TPO gene. Interestingly, transactivation experiments, carried out in HeLa cells on the Tg and TPO promoters, show that the converse situation is true for TTF-1, which activates Tg better than TPO (15a). Taken together with the observation that the binding of Pax-8 and binding of TTF-1 are mutually exclusive, it looks as if the thyroid cells could have evolved two different combinations of factors on the Tg and TPO promoters. The ratio of Tg and TPO transcription, which is always high in the thyroid, would be raised or lowered, depending on whether TTF-1 or Pax-8 is preferentially bound at the TATA box-proximal site in each of the two promoters. In this respect, it is interesting that both *Pax-8* and *TTF-1* are expressed, during thyroid development, several days before transcription of Tg and TPO, which are coordinately induced (34). While it is known that there is no delay in the appearance of the TTF-1 protein, similar data on the Pax-8 protein are lacking, leaving open the possibility that translation of an inactive Pax-8 mRNA is a decisive event in the induction of differentiation in the thyroid. Data on the appearance of the Pax-8 protein are necessary to address these questions further.

The finding that Pax-8 can bind to and activate transcription from two thyroid-specific promoters identifies, for the first time, natural promoters as targets for this type of regulatory molecule. The effect of Pax-8 on the promoters of Tg and TPO suggests that this protein could be important in the modulation of gene expression late in embryonal development and during adulthood. Such a modulation could be mediated by the interplay with the homeodomain-containing factor TTF-1, a particularly interesting observation in view of the presence of a homeodomain in some paired domain-containing genes (8, 31). Finally, the tissue distribution of TTF-1 and Pax-8 supports a combinatorial mechanism in the regulation of cell-type-specific transcription, since none of the two proteins is restricted to a single cell type, but their combination appears to be unique for the endodermally derived thyroid follicular cells. Inactivation of either gene should be able to demonstrate whether the simultaneous presence of Pax-8 and TTF-1 is necessary for thyroid differentiation.

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