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Cell-Type-Specific Expression of the Rat Thyroperoxidase Promoter Indicates Common Mechanisms for Thyroid-Specific Gene Expression

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A 420-bp fragment from the 5′ end of the rat thyroperoxidase (TPO) gene was fused to a luciferase reporter and shown to direct cell-type-specific expression when transfected into rat thyroid FRTL-5 cells. Analysis of this DNA fragment revealed four regions of the promoter which interact with DNA-binding proteins present in FRTL-5 cells. Mutation of the DNA sequence within any of these regions reduced TPO promoter activity. The *trans*-acting factors binding to these sequences were compared with thyroid transcription factor 1 (TTF-1) and TTF-2, previously identified as transcriptional activators of another thyroid-specific gene, the thyroglobulin (Tg) gene. Purified TTF-1 binds to three regions of TPO which are protected by FRTL-5 proteins. Two of the binding sites overlap with recognition sites for other DNA-binding proteins. One TTF-1 site can also bind a protein (UFB) present in the nuclei of both expressing and nonexpressing cells. TTF-1 binding to the proximal region overlaps with that for a novel protein present in FRTL-5 cells which can also recognize the promoter-proximal region of Tg. Using a combination of techniques, the factor binding to the fourth TPO promoter site was shown to be TTF-2. We conclude, therefore, that the FRTL-5-specific expression of two thyroid restricted genes, encoding TPO and Tg, relies on a combination of the same *trans*-acting factors present in thyroid cells.

Thyroid follicular cells are specialized epithelial cells which function to concentrate iodine and incorporate it into the thyroglobulin (Tg) molecule, which is subsequently broken down to release thyroid hormones. These processes require the synthesis of a set of cell-type-specific gene products, which include Tg, thyroperoxidase (TPO), and the receptor for the thyroid-stimulating hormone. All of these are present in the rat FRTL-5 cell line (4), which has been used as a model system to study thyroid-specific gene expression (9, 16, 31, 35, 48). It has been shown that restricted expression of Tg in the thyroid was due to transcriptional control (41). This cell-type-specific expression was shown to require the interaction of two thyroid-specific nuclear factors, thyroid transcription factor 1 (TTF-1) and TTF-2, and a ubiquitous factor, UFA, with the promoter (9, 48). TTF-1 is the most important determinant of thyroid specificity and has recently been cloned and shown to contain a homeobox motif (26). To gain further insight into the mechanisms controlling thyroid-specific gene expression, we have analyzed DNA sequences flanking the rat TPO gene and compared the *cis*-acting elements and *trans*-acting factors with those previously demonstrated to play a role in the Tg promoter. We report that a 420-bp promoter sequence from the rat TPO gene is able to confer thyroid-specific expression on a heterologous reporter. The *cis*-acting elements required for the expression of TPO have been tested in vitro for the binding of nuclear factors in thyroid and nonthyroid cells. The effects of mutations on factor binding demonstrate that TPO expression is mediated by the interaction of TTF-1, TTF-2, a novel thyroid-specific nuclear factor, and a ubiquitously expressed protein, UFB, with the TPO promoter.

MATERIALS AND METHODS

Isolation of TPO genomic sequences. A cDNA clone for rat TPO was isolated from a rat thyroid cDNA library cloned in λgt11, using a probe from the 3′ of the porcine TPO gene (gift of B. Rapaport). A single clone which showed considerable sequence homology to the porcine and human genes was subsequently used to screen a rat genomic library in λEMBL 3. A 5.8-kb *Eco*RI fragment derived from the genomic screen was subcloned into Bluescript KS- (Stratagene) to create prTPOG1.

Primer extension. Partial sequencing of the genomic insert in prTPOG1 enabled us to identify a region of homology with the 5′ end of human (32) and porcine (36) TPO mRNAs. This information was used to design oligonucleotide RTPO2 (5′-GCACAGCTGGTGTGTTCTTTGTGGCCTTAA-3′), which was subsequently used to identify the transcription start sites by primer extension analysis. A 40-ng sample of RTPO2 was end labelled with 15 pmol of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) by using polynucleotide kinase, extracted with phenol-chloroform, ethanol precipitated with 5 μg of yeast RNA, and resuspended in H_2O; 10 ng of kinase-labelled RTPO2 was mixed with 2 μg of either rat thyroid or liver RNA, prepared by guanidine thiocyanate lysis (7). The mixture was dried and resuspended in 10 μl of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 0.4 M NaCl, and 1 mM EDTA in 80% formamide], heated to 95°C for 3 min, and subsequently immersed in a 45°C water bath overnight. The nucleic acids

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were precipitated by the addition of 100 μl of 0.5 M ammonium acetate–10 mM EDTA and 100 μl of isopropanol and were washed with 70% ethanol. Reverse transcription was carried out in 20 μl of reaction mixture, using 30 U of Moloney reverse transcriptase (Pharmacia), in the presence of 0.5 mM deoxynucleoside triphosphates and 40 U of RNAsin (Promega) for 1 h at 37°C. The reaction was phenol-chloroform extracted, ethanol precipitated, and resuspended in 2 μl of formamide dye. The samples were loaded onto a 6% sequencing gel in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA), with a DNA sequence ladder as a molecular weight marker. The gel was fixed, dried, and exposed to Kodak X-Omat film overnight at −80°C.

Construction of TPO luciferase expression vectors. Standard DNA manipulations were carried out as described by Maniatis et al. (38). The luciferase coding region and simian virus 40 polyadenylation signal were excised from plasmid pJD206 (13) by digestion with *Hin*dIII and *Bam*HI. The resulting fragment was ligated to Bluescript KS- digested with *Hin*dIII and *Hin*cII and subsequently filled in at the *Bam*HI end with Klenow fragment. The ligated linear molecules were then circularized to give plasmid pBSLuc2. A 3.4-kb *Eco*RI-*Apa*L1 fragment of prTPOG1 was filled in with Klenow fragment and ligated to pBSLuc2, which had been digested with *Sma*I, to give the p3400TPOL expression vector. The 5′ deletions of the promoter were constructed as follows: a 2.0-kb *Pst*I fragment from p3400TPOL was subcloned into the *Pst*I site of pBSLuc2 to give p1900TPOL; p3400TPOL was cut with *Bam*HI and *Nco*I, treated with mung bean nuclease, and recircularized to give p1100TPOL; p800TPOL was constructed by *Pst*I-*Eco*RV digestion of p3400TPOL and fragment ligation to pBSLuc2 digested with *Pst*I and *Sma*I; and p420TPOL is a deletion of p3400TPOL created by the excision of a 3.0-kb *Sac*I fragment followed by religation of the expression vector. Fidelity of the constructs was verified by restriction mapping and sequencing of the junctions. The 420-bp TPO promoter was entirely sequenced in p420TPOL by using the Sequenase kit.

Promoter mutagenesis. The Am, Cm, Bmr, Bmm, and Bml mutations were constructed by using the Amersham mutagenesis system, based on the method of Taylor et al. (49), using single-stranded DNA from the p420TPOL vector as the starting template. Mutations were identified by sequencing of the promoter. Two independent clones were isolated for each mutant and subsequently assayed by transfection, thereby reducing the possibility that additional point mutations, which might arise in the luciferase gene during mutagenesis, contribute to a decrease in promoter activity. The Em, Pm, and Zm mutations were constructed by the polymerase chain reaction by the overlap extension method (27), using M13 and LUC-1 (5′-GGATAGAATGGCGCCGGG CCTTTCTTTATG-3′) oligonucleotides as flanking primers on the p420TPOL template. The amplified fragments were digested with *Sac*I and *Pst*I, subcloned into pBSLuc2, and checked by DNA sequencing.

DNase I footprinting. Nuclear extracts and purified TTF-1 were prepared as previously described (9). Purified TTF-1 homeodomain was obtained from a bacterial expression system (11). The footprinting probes were prepared by polynucleotide kinase labelling of p420TPOL digested at the *Eco*RI site (+75 bp), present in the polylinker of Bluescript, except that *Hin*dIII (−55 bp) was labelled in Fig. 4A. The plasmids were then digested with *Sac*I to yield fragments for analysis. DNase I footprinting reactions were performed in a 25-μl reaction volume as follows. For crude extracts, 5 to 30 μg of nuclear proteins was premixed with solution D (20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.9], 50 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol), 4 μg of yeast tRNA, and 0.1 mM ATP (pH 7.0). Purified TTF-1 was premixed with solution D, 12 μg of bovine serum albumin, 0.1% Nonidet P-40, and 2% polyvinyl alcohol. After addition of the probe, the reaction mixture was incubated for 45 min on ice. DNase I digestion was performed by the addition of 10 mM $MgCl_2$ and 2 mM $CaCl_2$ (final concentrations) and incubated for 1 min at 20°C; 0.3 to 12 ng (depending on the extract concentration) of DNase I was added, and the reaction mixture was incubated for 1 min; 200 μl of stop solution (20 mM Tris, 0.1 M NaCl, 1% sodium dodecyl sulfate [SDS], 50 μg of proteinase K per ml) was used to terminate DNase I digestion. Proteinase K treatment (30 min at 45°C) was followed by ethanol precipitation of the samples, which were resuspended in H_2O and lyophilized. The samples were then resuspended in 2 μl of formamide dye, heated for 3 min to 95°C, and run on a 6% 1× TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA) sequencing gel for 2 h. Gels were fixed, dried, and exposed to Kodak X-Omat film for 1 to 5 days with an intensifying screen.

Southwestern (DNA-protein) analysis. FRTL-5 and FRT nuclear proteins (100 μg) were electrophoresed through a 10% polyacrylamide–SDS gel (33) along with prestained molecular weight standards (Bethesda Research Laboratories). The proteins were electrotransferred overnight at 10 V to Hybond-N (Amersham) in 20 mM sodium phosphate buffer (pH 7.0). The filters were denatured in the presence of guanidium hydrochloride and renatured according to Vinson et al. (50). The oligonucleotides used as probes were K (Tg; 5′-TGACTAGCAGAGAAAACAAAGTGA-3′), Z (TPO; 5′-ACAAATACTAAACAAACAGAATGG-3′), and Zm (mutated Z; 5′-ACAAATAATCAACAAAACGAATGG-3′). Preparation of the double-stranded, polymerized oligonucleotide probes and subsequent binding to the filter and washing were done as described previously (50) except that the binding buffer consisted of 200 mM KCl, 40 mM HEPES, 0.2 mM EDTA, and 0.5 mM dithiothreitol. Filters were exposed to Kodak X-Omat film for 1 to 2 h at −80°C with the aid of an intensifying screen.

Mobility shift assay and methylation interference analysis. The conditions for the mobility shift assay were as previously described (48) except that in Fig. 9B the salt concentration was reduced to 75 mM KCl. Oligonucleotides Z, Zm, and K were as described for the Southwestern analysis. Oligonucleotide C (Tg) is 5′-CACTGCCCAGTCAAGTGTTC TTGA-3′, oligonucleotide P (TPO) is 5′-CTAAGCTTGAGT GGGCATCAGAGCATGGAG-3′, oligonucleotide Pm is 5′-CTAAGCTTGAGTCTCACGAGGAGCATGGAGTC-3′, oligonucleotide CT (TPO) is 5′-CTGTCTAAGCTTGAGTGG GCATCA-3′, and oligonucleotide C8A (Tg) is 5′-CACTGCA AAGTCAAGTGTTCTTGA-3′. In all competition experiments, the cold competitor was always preincubated with the extract for 10 min on ice prior to addition of the labelled probe.

For methylation interference analysis, the oligonucleotides were modified with dimethyl sulfate as described by Maxam and Gilbert (40) for 20 min on ice. For the preparative mobility shift, 2 to 4 ng (1×10^6 to 2×10^6 dpm) of modified oligonucleotides was incubated with 12 μg of poly[d(I-C)] and 20 μg of nuclear proteins. The gel was exposed wet for 1 h, and the bands were excised, eluted, and precipitated according to Maxam and Gilbert (40). Base elimination and strand scission reactions at adenines and

guanines (G>A) (40) were performed. The samples were then lyophilized three times, resuspended in 2 μl of formamide dye, heated to 95°C for 3 min, and loaded onto 20% sequencing gels in 0.5× TBE. Gels were exposed wet to Kodak X-Omat film for 1 to 4 days with an intensifying screen. Autoradiograms were quantitated by using the Macintosh Image 1.29 digital imaging and processing package (46).

Cell culture and transfection assays. FRTL-5, FRT, and Rat-1 cells were cultured as described previously (4, 48). For TPO promoter deletion and mutant analysis, 9-cm dishes were transfected by calcium phosphate-mediated transfection (24) with 10 μg of test constructs and 2 μg of Rous sarcoma virus (RSV) chloramphenicol acetyltransferase (CAT) to monitor for transfection efficiency. Cell extracts were prepared 48 h after transfection, and the CAT (23) and luciferase (13) activities were determined. The light units from the luciferase assay were normalized to 5% CAT conversion.

DNA sequences. The sequence data reported here have been deposited with the EMBL data library under accession number X61170.

RESULTS

Sequences from the 5′ flanking region of the TPO gene confer thyroid-specific expression on a heterologous reporter gene. The mRNA for rat TPO is present only in thyroid tissue or cell lines derived from rat thyroids (17). We have recently isolated a cDNA clone for rat TPO on the basis of its homology to a porcine TPO cDNA and subsequently TPO clones from a rat genomic library (17). The transcription initiation site was determined by primer extension analysis (Fig. 1A) and revealed two clusters of start sites (+1 to +5 and +7 to +9), analogous to those reported for the human gene (1, 32).

To determine whether the promoter region of TPO is able to confer cell-type-specific expression to a heterologous gene, we constructed a series of plasmids in which various lengths of 5′ genomic sequence flanking the TPO gene, including the transcription initiation sites, were fused to a luciferase reporter (13). These plasmids were transfected into FRTL-5 cells and control nonthyroid Rat-1 cells along with an RSV CAT reporter as an internal control for variations in transfection efficiency. TPO promoter fragments were inactive in the control Rat-1 cell line (Fig. 1B), which was able to express luciferase from a control cytomegalovirus promoter. All promoter fragments tested were active in FRTL-5 cells, although the longest fragment tested (3.4 kb) showed a markedly lower activity (Fig. 1B) than did any of the deletions. The smallest of the fragments assayed contains only 420 bp of TPO genomic sequence and retains cell type specificity, indicating that major determinants of thyroid specificity lie within this region. The entire DNA sequence of this fragment was determined and is presented in Fig. 1C.

Thyroid-specific and ubiquitous factors interact with the TPO promoter. To determine which nuclear factors might contribute to the FRTL-5-specific expression of the TPO promoter, DNase I footprints were performed on the genomic fragment contained in p420TPOL, using nuclear extracts prepared from either FRTL-5 cells or the control Rat-1 cells. Additionally, to verify whether any of the FRTL-5-enriched protections might be due to the previously identified factor TTF-1, purified TTF-1 from calf thyroids (9) was also used in footprinting experiments (Fig. 2A). Extracts from FRTL-5 cells protect four areas of the TPO minimal promoter, centered on −45 (C), −90 (Z), −100 (B), and −145 (A) bp. No protection of the A region was obtained with the Rat-1 extract, while TTF-1 and FRTL-5 extracts produced similar footprints (Fig. 2A), indicating that TTF-1 is likely to be the activity binding to this region of the promoter. In the C region, both FRTL-5 proteins and TTF-1 produce footprints, but the FRTL-5 extract shows an additional weak protection below the region protected by TTF-1 (Fig. 2A; compare the TL5 and TTF-1 lanes). Both FRTL-5 and Rat-1 proteins protect the B region of the promoter, resulting in footprints with a common lower border (Fig. 2A; compare the Rat-1 and TL5 lanes). However, the upper borders of the two footprints are different, as the FRTL-5 footprint extends into B′ (centered at −105), a region that is protected by purified TTF-1 (Fig. 2A; the asterisk between the Rat-1 and TL5 lanes indicates the bases protected only by the FRTL-5 extract). These data suggest that the FRTL-5 footprint could be due to the occupation of the B region by a protein also present in nonexpressing Rat-1 cells (UFB), together with TTF-1. This hypothesis is supported by data obtained with mutant promoters (see below). When the nucleotide sequences of the three TTF-1 binding sites in the TPO promoter (A, B′, and C) are compared with those previously identified in the Tg promoter, a common core sequence can be identified (Fig. 2B).

Footprints obtained with FRTL-5 extracts indicate the presence of an additional protein binding site in the Z region of the TPO promoter (Fig. 2A, TL5 lanes). Comparison of the FRTL-5 footprints with those obtained with Rat-1 nuclear extract suggests that the protein interacting with this region is specific to the expressing FRTL-5 cells.

A series of mutations in the TPO promoter was designed based on the protections observed in the footprinting analysis. A scheme of these mutations is presented in Fig. 3A. Each mutant was assayed for its ability to bind purified TTF-1 and the proteins present in FRTL-5 and Rat-1 nuclear extract by DNase I footprinting (Fig. 4). The transcriptional activities of mutant promoters (assayed by fusion to a luciferase reporter gene) cotransfected into FRTL-5 cells are shown in Fig. 3B; RSV CAT served as an internal control.

Binding of TTF-1 to the A region contributes to the full activity of the TPO promoter. The mutation in the A region (Am) of the TPO promoter substitutes the core consensus sequence for TTF-1 binding (Fig. 3A), thereby abolishing the binding of purified TTF-1 (Fig. 4A, lanes 2 and 7). The disappearance of the FRTL-5-specific footprint (Fig. 4A, lanes 3 and 8) suggests that TTF-1 is the FRTL-5 factor responsible for this protection in crude extracts. In vivo this mutation reduces promoter activity to 63% of the wild-type level (Fig. 3B), suggesting that this binding site makes a moderate contribution to the overall activity of the TPO promoter.

Mutation of the B region impairs TPO promoter activity. The B/B′ region is a complex site of DNA protein interactions on the TPO promoter, as indicated by the occurrence of three different but overlapping footprints in a small region. Three mutations were designed to interpret the contribution of TTF-1 and UFB to the FRTL-5 footprint and the promoter activity in FRTL-5 cells. The Bml mutation reduces the binding of purified TTF-1 (Fig. 4B, lanes 2 and 7), but the affinity of UFB appears unchanged in this mutant (Fig. 4B, compare lanes 4 and 9). The 5′ borders of the footprints obtained with FRTL-5 and Rat-1 proteins are now very similar (indicated by the asterisk in Fig. 4B, lanes 8 and 9), unlike the wild-type situation (Fig. 4B; indicated by the

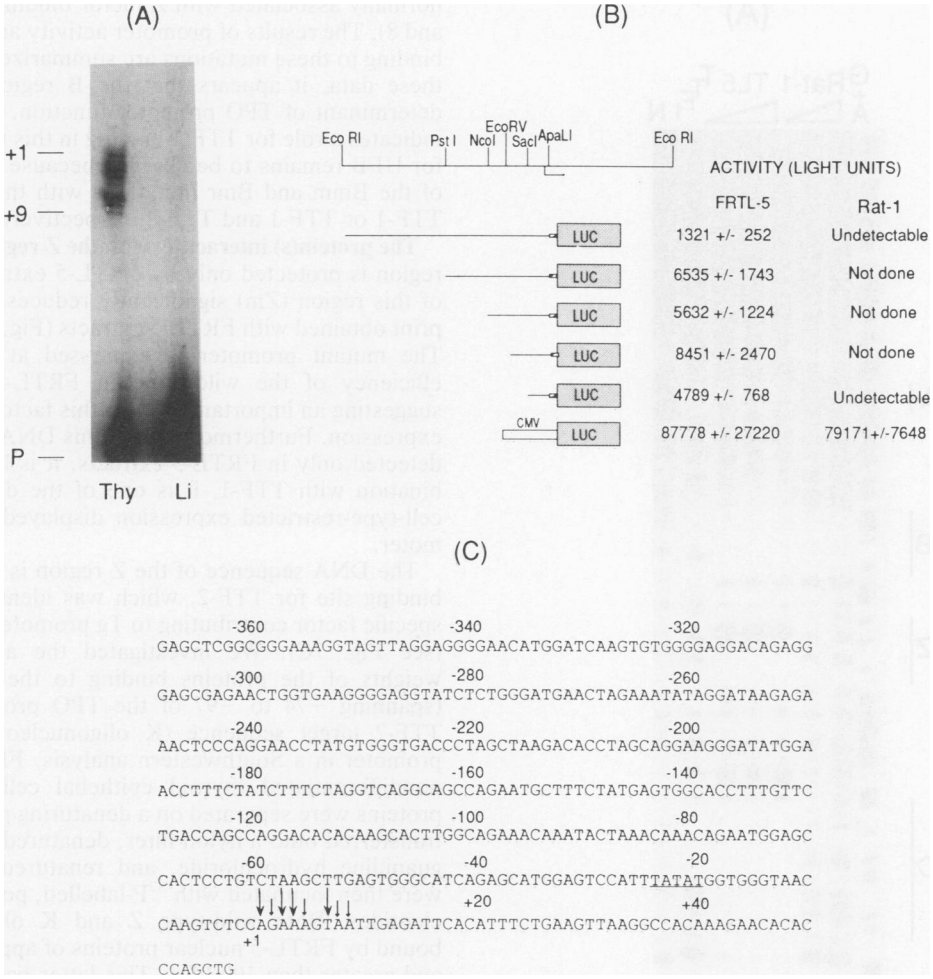

FIG. 1. (A) Primer extension analysis of rat TPO RNA. Rat thyroid or control liver poly(A)$^+$ RNA (2 μg) was hybridized to 10 ng of labelled oligonucleotide RTPO2, derived from the 5′ end of the rat TPO gene (see Materials and Methods) and extended with Moloney reverse transcriptase. The reaction mixtures were loaded onto a sequencing gel with a known DNA sequence ladder as a molecular weight marker. The migration of the primer, RTPO2, is indicated (P) at the left. Products of the extension reaction for liver (Li) and thyroid (Thy) RNAs are shown. The bases corresponding to the primer-extended products in thyroid are indicated at the left. (B) Schematic representation of the TPO promoter and TPO luciferase expression vectors. A restriction map representing the TPO genomic DNA insert in plasmid prTPOG1 is shown at the top; a scale bar is shown below. Beneath the restriction map, the length of 5′ genomic insert in each of the indicated luciferase expression vectors is represented as a line. CMV, cytomegalovirus. The luciferase gene is depicted as a shaded box, and the short stretch of polylinker sequence derived from Bluescript is depicted as a thin striped box. At the right is shown the luciferase activity (measured in light units, ± standard error of the mean) obtained with each expression vector when 10 μg was transfected into FRTL-5 or Rat-1 cells. RSV CAT was cotransfected with the luciferase reporters to normalize for any variability in transfection efficiency. (C) Sequence of the smallest genomic fragment capable of conferring tissue-specific expression on a luciferase reporter gene. The TATA sequence is underlined. The start sites of transcription are denoted with arrows. In accordance with the numbering for human gene (1, 32), the first start site is designated +1.

asterisk between lanes 3 and 4). This could indicate that in FRTL-5 extracts TTF-1 is able to occupy this site together with UFB, thus causing the extension of the footprint in the B′ region. On the other hand, footprinting experiments cannot dismiss a mutually exclusive interaction of UFB and TTF-1 with this region. Attempts to clarify this point by gel retardation experiments were unsuccessful because of the poor band shifts obtained even with partially purified UFB preparations. In FRTL-5 cells, the Bml mutation results in 63% of wild-type promoter activity (Fig. 3B). In the Bmm mutation, TTF-1 binding to the B′ region and UFB binding to the B site (Fig. 4C) are undetectable. Thus, the crucial bases for promoter recognition by UFB overlap with the TTF-1 core sequence present in this region (Fig. 2B). The Bmm mutant promoter displays only 34% of wild-type activity (Fig. 3B), illustrating the importance of this region for TPO promoter expression. Since this mutation abolished both TTF-1 and UFB binding, an additional mutation was designed to determine the contribution of UFB to TPO promoter activity. The Bmr mutation has a small effect on the affinity of TTF-1 for this region (Fig. 4D; compare lanes 2 and 7), as some bases in this region are weakly protected (Fig. 4D; indicated by the open circle adjacent to lane 7). However, this mutation causes a great reduction in UFB binding (Fig. 4D, lanes 4 and 9). The FRTL-5 extract no longer protects the B′ or B region efficiently (Fig. 4D, lanes 3 and 8), presumably because the TTF-1 concentration in the crude extract is too low. Additionally, a modest alteration in the affinity of the factor binding to the Z site occurred, observed as the reappearance of two bands which are

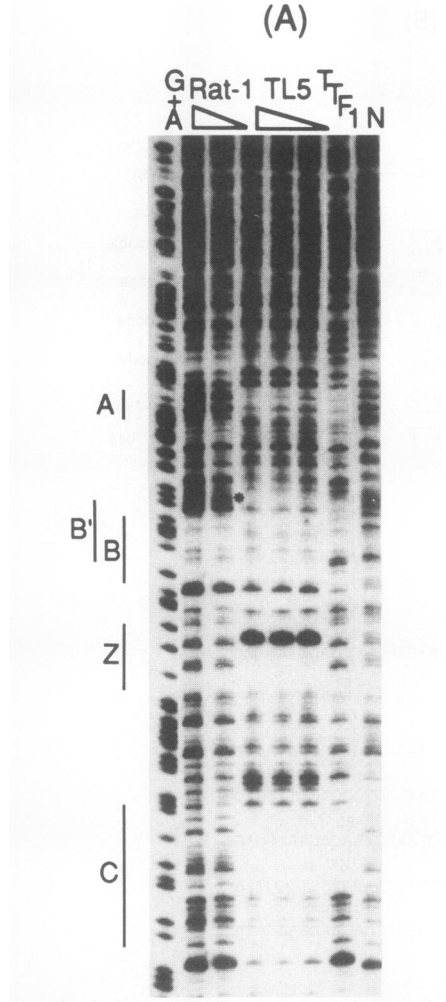

FIG. 2. Interaction of DNA-binding proteins with the TPO promoter. (A) DNase I footprint of the TPO promoter. p420TPOL was labelled at the *Eco*RI site within the polylinker (+75 relative to the transcription start site), using polynucleotide kinase and [γ-^{32}P] ATP. Lanes: G+A, Maxam-Gilbert sequencing reaction; Rat-1, protections given by Rat-1 nuclear proteins; TL5, protection in the presence of FRTL-5 nuclear proteins; TTF-1, protection obtained with affinity-purified TTF-1 from calf thyroids (9); N, control digestion pattern in the absence of added protein. The protected regions are depicted as lines at the left. The asterisk in the B′ region represents those additional bases protected from DNase I digestion by FRTL-5 proteins but not by Rat-1 proteins. (B) TTF-1 recognition sequences in the Tg and TPO promoters. The sequences are derived from the protections observed in DNase I footprinting and mutational analyses of both promoters. In the consensus sequence, uppercase letters refer to nucleotides conserved in six or seven of the binding sites and lowercase letters refer to four or five conservations.

normally associated with Z factor binding (compare lanes 3 and 8). The results of promoter activity and extents of factor binding to these mutations are summarized in Table 1. From these data, it appears that the B region is an important determinant of TPO promoter function. The Bml mutation indicates a role for TTF-1 binding in this region, while a role for UFB remains to be clarified because of the interference of the Bmm and Bmr mutations with the binding of either TTF-1 or TTF-1 and TTF-2, respectively.

The protein(s) interacting with the Z region is TTF-2. The Z region is protected only by FRTL-5 extracts, and mutation of this region (Zm) significantly reduces the DNase I footprint obtained with FRTL-5 extracts (Fig. 4E, lanes 3 and 8). The mutant promoter is expressed at only 20% of the efficiency of the wild type in FRTL-5 cells (Fig. 3B), suggesting an important role for this factor in TPO promoter expression. Furthermore, since this DNA binding activity is detected only in FRTL-5 extracts, it is likely that, in combination with TTF-1, it is one of the determinants of the cell-type-restricted expression displayed by the TPO promoter.

The DNA sequence of the Z region is very similar to the binding site for TTF-2, which was identified as a thyroid-specific factor contributing to Tg promoter transcription (48) (see Fig. 7C). We investigated the apparent molecular weights of the proteins binding to the Z oligonucleotide (spanning −74 to −97 of the TPO promoter) and to the TTF-2 target sequence (K oligonucleotide) from the Tg promoter in a Southwestern analysis. FRTL-5 and FRT (a nondifferentiated thyroid epithelial cell line) (3) nuclear proteins were separated on a denaturing polyacrylamide gel, transferred onto a nylon filter, denatured in the presence of guanidine hydrochloride, and renatured. The filter strips were then incubated with ^{32}P-labelled, polymerized oligonucleotides. Both wild-type Z and K oligonucleotides are bound by FRTL-5 nuclear proteins of approximately 60 kDa and greater than 100 kDa. This latter band appears to be a doublet with both probes and could represent two proteins or differential modification of a single protein (Fig. 5, probes Z and K in the TL5 lanes). These proteins do not recognize the Z mutant oligonucleotide (Fig. 5, probe Zm). The Z and K oligonucleotides are only weakly recognized by a high-molecular-weight protein present in the nondifferentiated FRT cells (Fig. 5, probes Z and K, FRT lanes). Thus, FRTL-5-enriched activities of apparent molecular sizes 60 and 100 kDa recognize the Z site of TPO and the K site of Tg.

The existence of cell-type-specific transcription factors of different molecular weights with similar DNA binding specificities has previously been described, for example in the octamer family (30, 47). Therefore, we investigated the electrophoretic mobility of protein-DNA complexes obtained by incubation of FRTL-5 nuclear extracts with either oligonucleotide Z or K (Fig. 6). No binding is observed to either oligonucleotide in Rat-1 nuclear extracts (Fig. 6, lanes 10 and 20) or in several other extracts from nonthyroid tissues (data not shown). When oligonucleotide Z is incubated with FRTL-5 nuclear proteins, one specific complex is observed and comigrates with the complex formed on oligonucleotide K (compare lanes 11 and 12 in Fig. 6). The K complex is competed for by addition of cold oligonucleotide

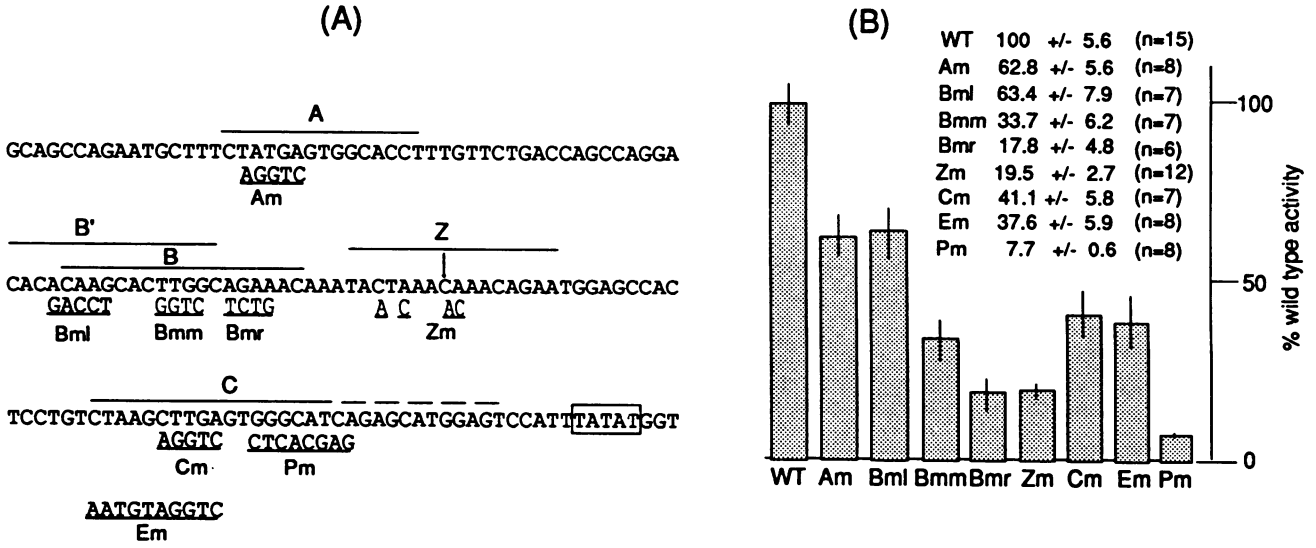

FIG. 3. Activities of mutant TPO promoters. (A) Scheme of mutations introduced into the protein binding sites in the TPO promoter. The regions protected from DNase I digestion in the footprinting analysis are shown as lines above the wild-type promoter sequence. The weak extension of the FRTL-5 footprint in the C region is shown as a dashed line. The arrow denotes the position of the DNase I-hypersensitive site associated with binding to the Z site. Mutations introduced into the TPO promoter are shown below the wild-type sequence, with the mutated bases underlined. (B) Effect of mutations on the activity of the TPO promoter in FRTL-5 cells. The mutated promoters were assayed by fusion to the luciferase reporter gene; 10 μg of luciferase reporter and 2 μg of RSV CAT were transiently expressed in FRTL-5 cells by calcium phosphate-mediated transfection (24). The activity of wild-type p420TPOL was taken as 100%, and the relative expression of the mutants was assessed (± standard error of the mean). n denotes the number of determinations for each mutant.

K or Z to the reaction (lanes 3 to 6) but not by the unrelated oligonucleotide C, which contains a TTF-1 binding site (lane 9). Similarly, the Z binding activity in FRTL-5 cells is competed for by addition of oligonucleotide Z or K (lanes 13 to 16) but not C (lane 19). Oligonucleotide Zm, containing a 4-bp mutation within the Z sequence, is not bound by FRTL-5 proteins (lane 23) and fails to compete for the complex formed with FRTL-5 extracts on both oligonucleotides Z and K (lanes 7 and 8; lanes 17 and 18). Taken together, the gel retardation assay and Southwestern experiment demonstrate that proteins of comparable molecular weight are able to bind both oligonucleotides.

Finally, the proteins present in FRTL-5 nuclear extracts were tested in a methylation interference assay to determine whether the equivalent bases were recognized within oligonucleotides Z and K. The data shown in Fig. 7 illustrate that methylation of bases at the corresponding positions within the two oligonucleotides interferes with factor binding. In particular, methylation of the G residues at position 8 or 17 in the lower strand of both oligonucleotides completely abolishes protein binding, presumably because these bases are being contacted by the protein (Fig. 7C). Together with the results of the oligonucleotide competition for the gel-retarded complexes, this result demonstrates that the FRTL-5 protein, which binds to the Z site, displays a similar binding specificity to TTF-2. However, which of the proteins identified by the Southwestern assay is responsible for the gel-retarded complex with this methylation interference pattern remains to be determined. As the Zm mutation severely impairs TPO promoter activity (Fig. 3B), we would conclude that TTF-2, which was previously defined as an FRTL-5-enriched activity that stimulates the Tg promoter by binding to the K sequence (9, 48), is also required for TPO promoter activity.

Mutational analysis of the TPO promoter-proximal region reveals the interaction of a novel FRTL-5 factor adjacent to the TTF-1 binding site. Mutation of the C region of the TPO promoter revealed that DNA binding to this region was more complex than it appeared on initial analysis. In Fig. 2A, a weak extension of the TTF-1 footprint toward the start site of transcription was observed only with FRTL-5 extracts. This finding suggests that another protein present in FRTL-5 cells, in addition to TTF-1, is capable of recognizing this region. The Cm mutation resulted in a different DNase I footprint obtained with purified TTF-1 (Fig. 8A, binding of TTF-1 to wild type and Cm). The same region is not protected by crude FRTL-5 extracts (Fig. 8A, TL5 lane), possibly because the site has a lower affinity for TTF-1 or because it overlaps with the binding site of TTF-2 in the neighboring Z region. Additionally, the DNase I-hypersensitive site at −60 is no longer visible (Fig. 8A, TL5 lanes). The displacement of TTF-1 from its wild-type binding site renders the weak footprint obtained with FRTL-5 extracts more obvious (Fig. 8A, TL5 lanes), extending from −55 to a weak but reproducible DNase I-hypersensitive site at −29 (indicated by an arrow in Fig. 8A). The Cm mutant displays 41% residual promoter activity (Fig. 3B), indicating that TTF-1 binding to its cognate sequence is required for full promoter activity. However, the role of the additional TTF-1 binding site and the prominent protection obtained with FRTL-5 extracts were investigated in greater detail by the creation of additional mutations.

Mutation Em substitutes five extra bases in addition to those altered by the Cm mutation (Fig. 3A). No protection of the C region is obtained with purified TTF-1 (Fig. 8B, TTF-1 binding to Em), and the DNase I-hypersensitive site at −60 is absent (Fig. 8B, TL5 binding to Em). As with the Cm mutation, the binding of the additional factor present in FRTL-5 nuclear extracts to the region −55 to −29 is clearly visible (Fig. 8B, Em probe, TL5 lanes). As in the wild-type

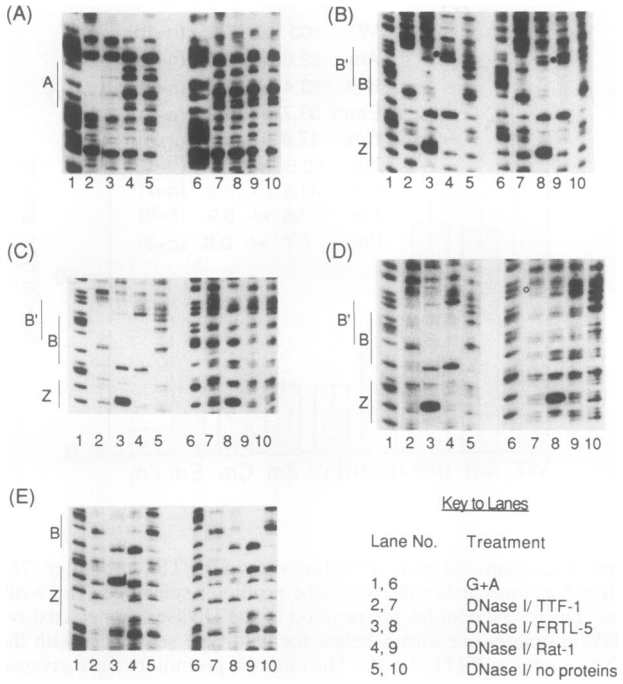

FIG. 4. Effect of mutations on the binding of *trans*-acting factors to the TPO promoter A, B'/B, and Z regions. Probes were prepared by kinase labelling at the −55 *Hin*dIII site in the TPO promoter (A) or the +75 *Eco*RI site within the polylinker of pTPOL vectors (B to E). For each experiment, purified TTF-1 (0.1 to 1 μl) and nuclear proteins from FRTL-5 cells (5 to 25 μg) or Rat-1 cells (2.5 to 15 μg) were titrated over wild-type and mutant TPO promoters, which were subsequently digested with DNase I and fractionated on the same 6% sequencing gel in 0.5× TBE. For each mutation, the digestion patterns of the wild-type (lanes 1 to 5) and mutant (6 to 10) promoters are shown. Outside of the regions presented, no other changes were observed in the DNase I digestion pattern. The wild-type protections for each region are depicted on the left as lines. Lanes for all experiments: 1 and 6, the Maxam-Gilbert G+A sequencing reactions of the wild-type and mutant promoter sequences, respectively; 2 and 7, binding of purified TTF-1 to wild-type and mutant promoters, respectively; lanes 3 and 8, interaction of FRTL-5 proteins with wild-type and mutant promoters; 4 and 9, binding of Rat-1 nuclear proteins to each probe; 5 and 10, control digestion patterns of the wild-type and mutant probes, respectively. (A) The Am mutation abolishes TTF-1 binding and the FRTL-5 footprint in the A region. (B) The Bml mutation reduces TTF-1 binding to B', resulting in similar FRTL-5 and Rat-1 footprints. The asterisk between lanes 3 and 4 highlights the different 5' borders of FRTL-5 and Rat-1 protections. The same region is marked on the mutant promoter between lanes 8 and 9 where the 5' borders become very similar. (C) The Bmm mutation abolishes both TTF-1 and UFB interactions with the B'/B region. (D) The Bmr mutation greatly reduces the interaction of UFB and has lesser effects on the binding of TTF-1 and the factor binding to the Z region. The open circle between lanes 6 and 7 marks the partial protection of bases in the B' region by purified TTF-1. (E) Mutation of the Z region abolishes binding of the factor present in FRTL-5 extracts.

and Cm promoters, the Rat-1 extract does not protect this region (Fig. 8C), confirming that the protein responsible for this footprint is present only in FRTL-5 cells. The activity of the Em mutant promoter in FRTL-5 cells (37%) is not significantly different from that of the Cm mutant (Fig. 3B), thereby confirming the extent of TTF-1-mediated transcription stimulation from the C site previously assessed by the Cm mutation.

TABLE 1. Effects of mutations in the B'/B/Z region of the TPO promoter on recognition by three DNA-binding proteins and the observed promoter activity in FRTL-5 cells

Mutation	Affinity			Activity (%)
	TTF-1/B'	UFB/B	TTF-2/Z	
Wild type	++++	++++	++++	100
Bml	++	++++	++++	63.4
Bmm	−	−	++++	33.7
Bmr	++	−	+++	17.8
Zm	++++	++++	−	19.5

[a] The affinity of each of the DNA-binding proteins for the mutant promoters was determined by titration analysis. ++++, wild-type affinity; +++, twofold or less reduction in affinity; ++, affinity reduced two- to fourfold; +, affinity reduced four- to eightfold; −, eightfold or greater reduction in affinity.

To investigate the additional FRTL-5 footprint overlapping with the natural TTF-1 protection of the C region, Pm, an 8-bp substitution adjacent to the TTF-1 core sequence, was created (Fig. 3A). This mutation reduced the binding of purified TTF-1 at least sevenfold (Fig. 8B, compare protection of the wild-type probe at the lowest TTF-1 concentration with that at the highest on Pm, 7.5-fold excess). A significantly weaker TTF-1 protection was obtained with FRTL-5 extracts, although the DNase I-hypersensitive site at −60 is suggestive of some residual binding activity (Fig. 8B, TL5 lanes). Additionally, no protection of the −55 to −29 region or of the DNase I-hypersensitive site at −29 (indicated by the arrow) is observed with the FRTL-5 extract, suggesting that the binding of the additional factor is severely impaired (Fig. 8B, TL5 lanes). The effect of this mutation on promoter activity in FRTL-5 cells (8% of the wild-type-level) is dramatic and indicates a major role for the additional FRTL-5 factor in determining the transcriptional activation mediated by the C region of the TPO promoter.

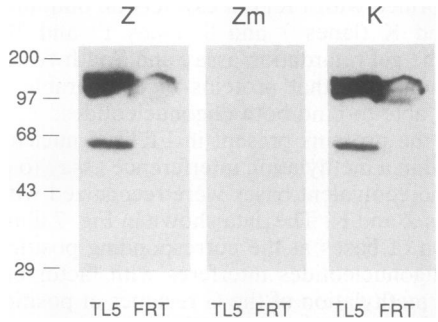

FIG. 5. Southwestern blot analysis of FRTL-5 nuclear proteins. Two 100-μg samples each of FRT (FRT) and FRTL-5 (TL5) nuclear proteins and prestained molecular weight markers were separated on a denaturing protein gel. Proteins were electrotransferred to nylon membranes, and denatured in the presence of guanidine-HCl, and renatured. The two strips were subsequently incubated with the ^{32}P-labelled oligonucleotides: Z (Z oligonucleotide derived from the TPO promoter) and Zm (the Zm oligonucleotide containing the 4-bp substitution). The filter incubated with the Z oligonucleotide was subject to a second round of denaturation and renaturation and controlled for effective signal removal (no signal was detected after overnight exposure). This filter was subsequently rehybridized to the K oligonucleotide from the Tg promoter (third panel), which binds the activity previously defined as TTF-2 (9, 48). The autoradiographs were exposed for 1 to 2 h. The relative positions (kilodaltons) of the prestained molecular weight markers are indicated at the left.

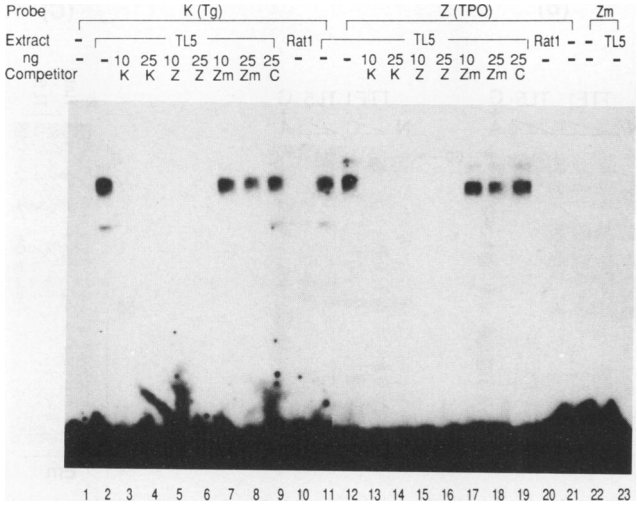

FIG. 6. Gel mobility shift assay with the TPO Z and the Tg K oligonucleotides. Double-stranded ^{32}P-labelled oligonucleotides (0.4 ng per lane) were incubated with 5 μg of FRTL-5 or Rat-1 nuclear proteins and 10 or 25 ng of competitor oligonucleotide K, Z, Zm, or C (see Materials and Methods), as indicated above the lanes. In lanes 1 to 11, oligonucleotide K from the Tg promoter forms a complex previously defined as TTF-2 (9, 48). Lanes 12 to 21, complex formed with oligonucleotide Z from the TPO promoter; lanes 22 and 23, inability of oligonucleotide Zm to bind FRTL-5 proteins.

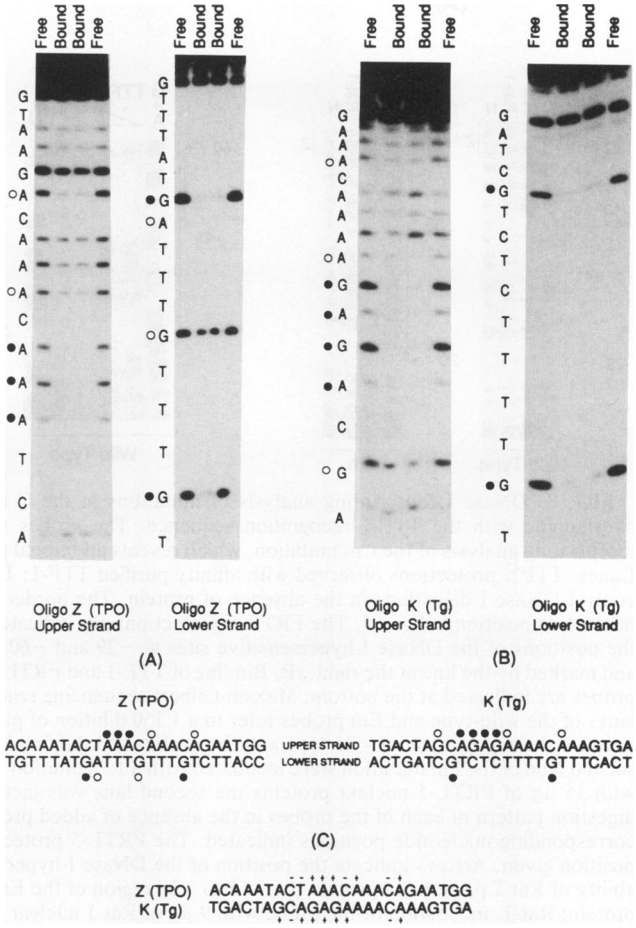

FIG. 7. Methylation interference analysis of proteins interacting with the Z region of the TPO promoter (A) and the K region of the Tg promoter (B). ^{32}P-labelled, dimethyl sulfate-modified oligonucleotides were incubated with 20 μg of FRTL-5 nuclear extract and resolved on a native gel as described in Materials and Methods. The bands corresponding to bound complexes and free DNA were eluted from the gel and cleaved at the modified residues. The cleavage products were resolved by electrophoresis through 20% denaturing gels and visualized by autoradiography. The sequence of the bases is indicated at the left. Filled circles define the bases whose modification reduces the proportion of DNA in the bound fraction by 3-fold or more; open circles define bases whose effect is to reduce the binding by 1.5 to 3-fold. Beneath the autoradiographs, the sequences of both strands are summarized, together with those bases whose methylation interferes with binding. (C) Sequence alignment of oligonucleotides Z and K, illustrating the near identity of the interference patterns. The upper strand of each oligonucleotide is depicted. Crosses mark bases at which methylation on the top strand interferes with binding; a dash indicates that methylation of the lower-strand complement inhibits binding.

Interestingly, the promoter-proximal regions of TPO and Tg show a much greater sequence homology when compared with the other TTF-1 target sites (Fig. 9A, CT and C), having perfect conservation of a TGCCC motif 5′ to the core TTF-1 recognition sequence. This conserved motif is altered by the Pm mutation in TPO (Fig. 9A). This observation prompted us to test whether an FRTL-5 protein whose binding was affected by the Pm mutation might also interact with this sequence in the Tg promoter. A gel retardation assay was performed using oligonucleotide P, spanning −60 to −31 of the TPO promoter (Fig. 9B). We expected to observe two DNA-protein complexes with this oligonucleotide, as it contains a TTF-1 binding site in addition to the TGCCC motif. However, of the two complexes observed with FRTL-5 proteins (Fig. 9B), the faster one is nonspecific, since it is not competed for by a large excess of the cold P oligonucleotide and was not studied further. The remaining complex is specific, since it is abolished by the addition of excess cold competitor (Fig. 9, lanes 3 and 4) but not by the addition of the Pm oligonucleotide, which contains the Pm mutation (lanes 5 and 6). Two shorter oligonucleotides derived from the TPO (CT, lanes 7 and 8) and Tg (C, lanes 9 and 10) promoters, containing the TGCCC motif, are also efficient competitors for this complex. However, oligonucleotide C8A, derived from the Tg promoter, which substitutes two of the conserved bases and does not impair TTF-1 binding (52), is a poor competitor and fails to abolish the complex even at 250-fold excess (lanes 11 and 12). In Rat-1 nuclear extracts, no binding to the probe was observed (lane 13). Although oligonucleotide P contains two binding sites, only one specific retarded complex was detected by this assay, probably because the retarded band is composed of two different complexes of a similar electrophoretic mobility. The inability of oligonucleotide C8A to compete completely for complex formation is presumably due to the mutation which disrupts the binding of only one of these two proteins.

The TPO TATA element limits promoter activity. The Tg and TPO promoters are remarkably similar in organization. Factors present in the nuclei of thyroid cells bind to both promoters at similar positions (see Fig. 11). However, the TPO promoter is approximately an order of magnitude less active than the Tg promoter in FRTL-5 cells (unpublished observations). Such a difference could be achieved by various mechanisms, such as the contribution to promoter

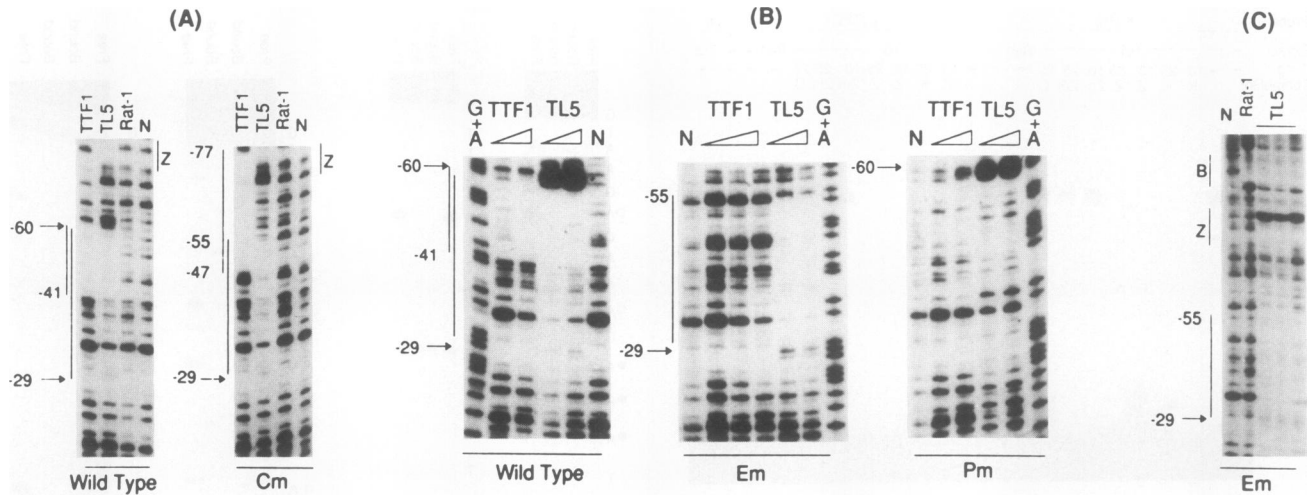

FIG. 8. DNase I footprinting analysis of mutations in the C region of TPO, demonstrating that an additional protein binds to a site overlapping with the TTF-1 recognition sequence. The probes were all labelled with [γ-^{32}P]ATP at (+75) as previously. (A) DNase I footprinting analysis of the Cm mutation, which reveals additional protein binding sites. The identity of each probe is indicated at the bottom. Lanes: TTF1, protections observed with affinity purified TTF-1; TL5, FRTL-5 nuclear proteins; Rat-1, Rat-1 nuclear proteins; N, indicates control DNase I digestions in the absence of protein. The borders of the TTF-1 protections are shown at the left, with the corresponding nucleotide position indicated. The FRTL-5 protections are indicated by the outer lines, with the nucleotide position shown. Arrows indicate the positions of the DNase I-hypersensitive sites at −29 and −60, observed with the FRTL-5 extract. The 3′ end of the Z region is visible and marked by the line at the right. (B) Binding of TTF-1 and FRTL-5 proteins to the Em and Pm mutations of the TPO promoter. The labelled probes are indicated at the bottom. Maxam-Gilbert sequencing reactions performed on each of the probes are denoted G+A. The first TTF-1 lanes of the wild-type and Em probes refer to a 1:150 dilution of purified TTF-1 homeodomain. A 1:50 dilution of purified homeodomain was used for the second lane of wild-type and Em probes and the first lane of TTF-1 for the Pm probe. The third lane of the Em mutation and the second lane of the Pm mutation were incubated with a 1:20 dilution of purified homeodomain. For each probe, the first TL5 lane was incubated with 15 μg of FRTL-5 nuclear proteins the second lane was incubated with 30 μg of FRTL-5 nuclear proteins. N refers to the DNase I digestion pattern of each of the probes in the absence of added protein. The borders of the TTF-1 protections are shown at the left, with the corresponding nucleotide positions indicated. The FRTL-5 protections are indicated by the outer lines, with the corresponding nucleotide position given. Arrows indicate the position of the DNase I-hypersensitive sites at −29 and −60, observed with the FRTL-5 extract. (C) In ability of Rat-1 proteins to protect the −55 to −29 region of the Em promoter. Lanes: N, DNase I digestion pattern in the absence of added protein; Rat-1, incubation of the probe with 9 μg of Rat-1 nuclear proteins prior to DNase I digestion (the control UFB protection on the B region is visible and denoted by a line at the left); TL5, probe preincubated with 10 μg of FRTL-5 extract prior to DNase I digestion. The protection by TTF-2 on the Z region is depicted at the left. The protection of the −55 to −29 region is indicated by the line, with the nucleotide positions shown.

activity by the ubiquitous factors or the affinity of any of the factors for their cognate binding sites. Another possibility is that the two TATA elements are not equivalent, since substitution of residues within TATA boxes has been demonstrated to affect promoter activity (10, 21, 42). Notably, promoters with poor TATA elements start transcription at a series of sites (5, 6, 39), as has been observed for both the rat (this report) and human (1, 32) TPO promoters. Conversely, the rat Tg gene initiates transcription at a single site (41). We therefore substituted the Tg TATA box sequence for that present in the wild-type TPO promoter (Fig. 10A). This mutation increased the expression of the TPO promoter threefold in FRTL-5 cells (Fig. 10B), suggesting that a poor TATA element limits the expression of the TPO promoter.

DISCUSSION

In this study, we have investigated the *cis*-acting elements and *trans*-acting factors which lead to the tissue-specific expression of the rat TPO promoter in the thyroid cell line FRTL-5. Deletion analysis revealed that a 420-bp fragment of the TPO promoter is sufficient to confer cell-type-specific expression on a heterologous reporter gene. Additionally, the longest (3.4 kb) of the TPO promoter fragments tested was less active than shorter fragments of the promoter, suggestive of some negative element modulating TPO promoter activity in FRTL-5 cells. Alternatively, the longer plasmid may contain sequences able to form structures which are refractory to TPO expression in transient expression assays. This hypothesis was proposed by Kikkawa et al. (31), who observed that transient transfection of vectors containing 4.1 or 3.1 kb of 5′ sequence from the human promoter gave no expression in FRTL-5 cells, yet the same plasmids were expressed in stable FRTL-5 clones.

Two groups have reported that the human TPO promoter is unable to confer tissue-specific transcription on reporter genes (16, 31), although the 1.3-kb segment of the human TPO promoter was specifically activated in primary dog thyroid cell cultures (1). However, in this report we demonstrate that the rat TPO gene is appropriately expressed in rat FRTL-5 cells but does not transcribe in control rodent cells. Additionally, the promoter is appropriately downregulated in a number of dedifferentiated rat thyroid cell lines, obtained by transformation with retroviral oncogenes (18). These discrepancies might be due to the use of different heterologous systems and reflect species differences in the promoter organization or the availability of the relevant *trans*-acting factors.

Analysis of the elements required for promoter activity illustrate that the TPO and the Tg promoters display a remarkable conservation in the organization of the binding sites for TTF-1 and TTF-2, shown schematically in Fig. 11.

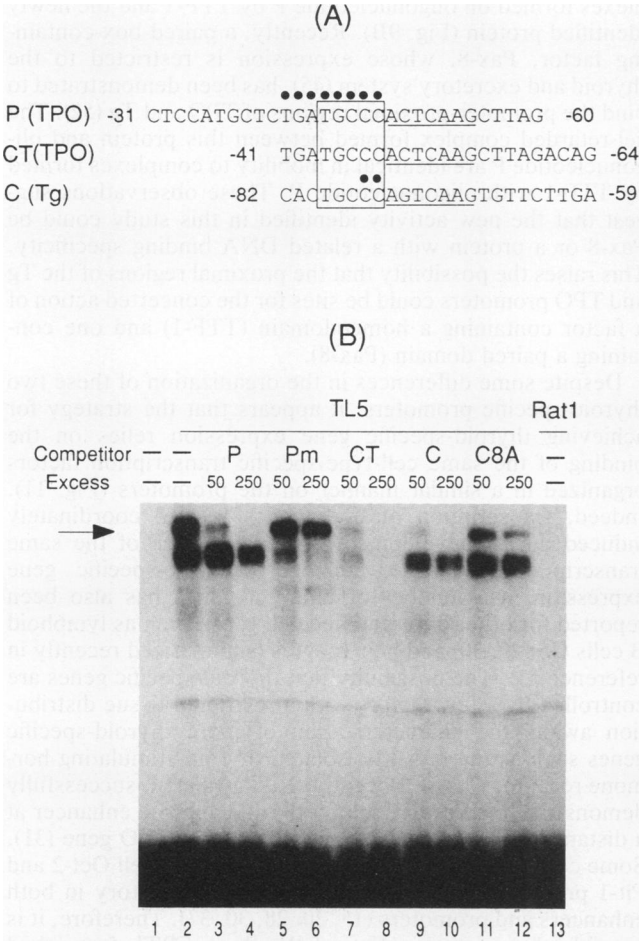

FIG. 9. Evidence that TPO and Tg promoters contain a common sequence motif able to interact with FRTL-5 proteins. (A) DNA sequence of the oligonucleotides used in the gel retardation experiment. The DNA sequence of oligonucleotide P, which spans the promoter-proximal region of TPO, is shown at the top. Circles represent the nucleotides substituted by the Pm mutation. CT is a shorter oligonucleotide derived from the promoter-proximal region of TPO but is centered on the TTF-1 core motif. C is an oligonucleotide centered on the promoter-proximal TTF-1 binding site of the Tg promoter. Circles represent the base substitutions in oligonucleotide C8A, used for the gel retardation experiment in panel B. The TGCCC motif is boxed, and the TTF-1 core consensus sequence is underlined. Numbers at the ends of each oligonucleotide refer to the nucleotide position within the promoter from which they were derived. Note that the bottom-strand sequence is shown for the TPO promoter sequences, whereas the top strand of the Tg sequence is shown. (B) Gel retardation assay using oligonucleotide P, revealing an FRTL-5 complex unable to interact with the Pm oligonucleotide. Oligonucleotide P was labelled with [γ-^{32}P]ATP by using polynucleotide kinase; 3 μg of nuclear proteins from FRTL-5 or Rat-1 cells was incubated with the probe in the absence or presence of 50- and 250-fold molar excesses of various competitor oligonucleotides. Complexes were separated by electrophoresis on native TBE gels and visualized by autoradiography. Lanes: 1, no proteins; 2, complexes formed with FRTL-5 proteins in the absence of competitor DNA; 3 to 12, complexes of FRTL-5 proteins in the presence of 50- and 250-fold molar excesses of the competitors indicated above the lanes (the arrow denotes the FRTL-5-specific complex affected by mutation); 13, Rat-1 proteins in the absence of competitor.

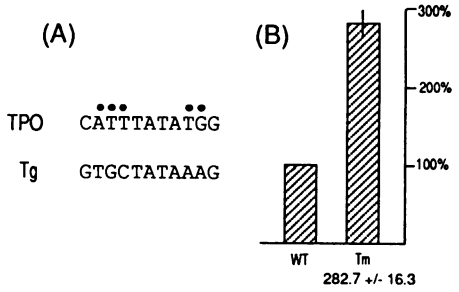

FIG. 10. Evidence that substitution of the TATA element of the TPO promoter for the Tg sequence stimulates TPO promoter activity. (A) Alignment of sequences of the TPO and Tg TATA elements. Bases marked with circles were substituted for the bases in the Tg promoter to give the Tm mutation. (B) Relative activities of wild-type and Tm TPO promoters, assessed by transient transfection of luciferase reporter genes in FRTL-5 cells. Luciferase activity was determined from extracts expressing equivalent amounts of CAT activity from the cotransfected RSV CAT reporter.

TTF-1 can bind three times to both promoters, at positions −50, −105, and −145 in TPO, whereas in Tg the binding is observed at −70, −125, and −150. TTF-2 binds each promoter once, with the binding site centered at −90 in TPO and −95 in Tg. A third thyroid-specific factor which interacts with the promoter-proximal element in TPO was identified in this study. Competition experiments suggest that it is also capable of recognizing the equivalent region of Tg. Furthermore, in both cases, the binding site of this new factor overlaps or is very close to the proximal TTF-1 binding site. The relevance of TTF-1 and TTF-2 binding to both promoters is supported by the effect of mutation of their target sequences (this study; 9, 48) and by transient transfection experiments which demonstrate that TTF-1 is able to activate transcription from the TPO and Tg promoters in nonthyroid cells in a manner dependent on the integrity of the proximal TTF-1 binding site (12). Apart from these relevant structural homologies, the TPO promoter differs in several aspects from that of Tg. One such difference appears to be the relative efficacy with which the protein binding sites stimulate transcription of the two promoters. The TPO A region, which interacts with TTF-1 alone, is the least effective region at stimulating transcription (63% residual

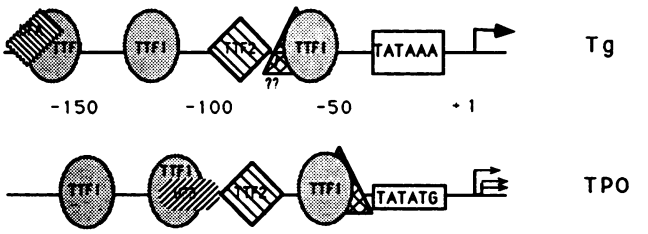

FIG. 11. Schematic representation of Tg and TPO promoter occupancy by the DNA-binding proteins present in rat FRTL-5 cells. Some of the factors interacting with the Tg promoter have previously been described (9, 48); the one represented as a triangle was identified in this study. Symbols: ovals, TTF-1 binding; diamond, TTF-2 binding; triangle, the factor affected by the Pm mutation. The ubiquitous factors UFA and UFB are also represented. The TATA sequences of the two promoters are not equivalent (this study) and are boxed. The transcription start sites of the two promoters are represented by arrows.

activity when binding is severely impaired) of the promoter. The equivalent region of the Tg promoter is bound by both TTF-1 and the ubiquitous factor UFA, and some mutations within this region reduce promoter activity to as little as 4% (9, 48). Conversely, the TPO B region can bind both TTF-1 and a ubiquitous factor, UFB, and is essential for full activity, while the corresponding region of Tg, which binds only TTF-1, is almost dispensable (9, 35, 48). Therefore, the two most distal TTF-1 binding sites of the two promoters appear to differ in importance. Interestingly, although the positions of the binding sites are inverted, in both cases the most effective mutations occur where TTF-1 interacts with a ubiquitous factor.

Interestingly, the B region of the TPO promoter contains a TTGGCA motif, which is changed by the Bmm mutation and destroys UFB binding. This motif constitutes a perfect NF-1 half-site, similar to those identified previously in other cell-type-specific elements such as the albumin promoter (43) and the adipocyte aP2 enhancer (25) as well as the cyclic AMP-responsive region of the human proenkephalin gene (8). Footprinting experiments carried out with the purified DNA binding domain of NF-1 (kind gift of P. Van der Vliet) indicate that it recognizes this site with high affinity (data not shown) and suggests that UFB is a member of the family of NF-1-like factors (14, 22). Although mutational analysis of this region is suggestive of a role for both UFB and TTF-1 in stimulating promoter activity (Table 1), the relative efficacy of either remains to be determined.

The differences in the type and positioning of the ubiquitous factor may be related to the distinct mode of regulation of these two thyroid-specific promoters. The mechanisms governing the regulation of the two promoters in response to thyroid-stimulating hormone appear to be different: TPO responds in a rapid, protein synthesis-independent manner, whereas the Tg response is slow and cycloheximide sensitive (19, 29). Such a difference may reside in unidentified *trans*-acting factors interacting with either promoter or may be due to the differences in binding site affinity or organization for any of the factors that we have identified. Additionally, whereas Tg is a very strong thyroid-specific promoter with a single transcription initiation site (41), TPO is relatively weak in its expression and transcription initiates at a cluster of bases (this study; 1, 32). In this report, we have demonstrated that some difference in the potency of the two promoters appears to reside in the TATA element, since substitution of the Tg sequence into the TPO promoter increases transcriptional efficiency.

Both TPO and Tg promoters rely on the proximal promoter region to stimulate transcription. This study revealed the presence of an additional factor, in FRTL-5 extracts, which can recognize this same region in both promoters. Mutational analysis of the TPO promoter indicates that this additional factor is relevant for transcriptional activity, as the Cm or Em mutations, which affect only TTF-1 binding, have 40% residual activity, while the Pm mutation, which affects both TTF-1 and the newly identified factor, reduces promoter activity to 8% of the wild-type level. The proximal regions of the TPO and Tg promoters show a much greater sequence homology when compared with the other TTF-1 target sites [Fig. 9A, CT(TPO) and C(Tg)], having conserved a TGCCC motif 5′ immediately adjacent to the TTF-1 binding site. Interestingly, we had previously described a mutation of the Tg promoter which changed two nucleotides of the TGCCC motif (pTACAT 13) and resulted in a reduction of Tg promoter activity, although TTF-1 binding was unaffected (48). Attempts to develop a gel retardation assay for the new factor identified as interacting with this region of TPO have been hampered by the comigration of the complexes formed on oligonucleotide P by TTF-1 and the newly identified protein (Fig. 9B). Recently, a paired-box-containing factor, Pax-8, whose expression is restricted to the thyroid and excretory system (45), has been demonstrated to bind the promoter-proximal regions of TPO and Tg (52). The gel-retarded complex formed between this protein and oligonucleotide P are identical in mobility to complexes formed by TTF-1 with oligonucleotide P. These observations suggest that the new activity identified in this study could be Pax-8 or a protein with a related DNA binding specificity. This raises the possibility that the proximal regions of the Tg and TPO promoters could be sites for the concerted action of a factor containing a homeodomain (TTF-1) and one containing a paired domain (Pax-8).

Despite some differences in the organization of these two thyroid-specific promoters, it appears that the strategy for achieving thyroid-specific gene expression relies on the binding of the same cell-type-specific transcription factors organized in a similar manner on the promoters (Fig. 11). Indeed, transcription of both genes is also coordinately induced during development (34). The usage of the same transcription factors to achieve cell-type-specific gene expression in a number of different genes has also been reported for other differentiated cell types such as lymphoid B cells (15, 20, 30) and hepatocytes (summarized recently in reference 51). The possibility that thyroid-specific genes are controlled by a few factors with a restricted tissue distribution awaits the characterization of more thyroid-specific genes such as the recently isolated thyroid-stimulating hormone receptor (2, 44). Recently, Kikkawa et al. successfully demonstrated the presence of a thyroid-specific enhancer at a distance of 5.5 kb upstream of the human TPO gene (31). Some cell-type-specific factors, such as the B-cell Oct-2 and Pit-1 proteins, have been shown to be stimulatory in both enhancers and promoters (15, 20, 28, 30, 37). Therefore, it is possible that a combination of the three FRTL-5-enriched factors described here may also be responsible for the activity of the human TPO enhancer.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Recently, Mizuno et al. (K. Mizuno, F. J. Gonzalez, and S. Kimura, Mol. Cell. Biol. **11**:4927–4933, 1991) demonstrated that TTF-1 does indeed interact with the thyroid-specific enhancer identified in the human TPO gene, thus confirming a general role for TTF-1 in the control of thyroid-specific transcription.

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