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The Paired Domain-containing Factor Pax8 and the Homeodomain-containing Factor TTF-1 Directly Interact and Synergistically Activate Transcription*

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Pax genes encode for transcription factors essential for tissue development in many species. Pax8, the only member of the family expressed in the thyroid tissue, is involved in the morphogenesis of the gland and in the transcriptional regulation of thyroid-specific genes. TTF-1, a homeodomain-containing factor, is also expressed in the thyroid tissue and has been demonstrated to play a role in thyroid-specific gene expression. Despite the presence of Pax8 and TTF-1 also in a few other tissues, the simultaneous expression of the two transcription factors occurs only in the thyroid, supporting the idea that Pax8 and TTF-1 might cooperate to influence thyroid-specific gene expression. In this report, we describe a physical and functional interaction between these two factors. The fusion protein GST-Pax8 is able to bind TTF-1 present in thyroid or in non-thyroid cell extracts, and by using bacterial purified TTF-1 we demonstrate that the interaction is direct. By co-immunoprecipitation, we also show that the interaction between the two proteins occurs *in vivo* in thyroid cells. Moreover, Pax8 and TTF-1 when co-expressed in HeLa cells synergistically activate Tg gene transcription. The synergism requires the N-terminal activation domain of TTF-1, and deletions of Pax8 indicate that the C-terminal domain of the protein is involved. Our results demonstrate a functional cooperation and a physical interaction between transcription factors of the homeodomain-containing and of the paired domain-containing gene families in the regulation of tissue-specific gene expression.

Tissue-specific transcriptional regulation is often mediated by a complex of cis-acting elements. The vast majority of the promoters of genes expressed in a cell type-specific fashion contains a variety of recognition sequences for tissue-specific and ubiquitous transcription factors. It is well known that tissue-specific transcriptional regulation is mediated by a set of transcription factors whose combination is unique to the cell type. Thyroid follicular cells, the most abundant cell population of the thyroid gland, represent a useful model system to elucidate the mechanism operating in the establishment and maintenance of cell type-specific expression. Thyrocytes are responsible for thyroid hormone synthesis and are characterized by the expression of a specific set of genes such as thyroglobulin (Tg)[1] and thyroperoxidase (TPO), which are exclusively expressed in this cell type (1, 2), and by the expression of genes expressed only in a few tissues other than the thyroid, such as the thyrotropin-stimulating hormone receptor and the sodium/iodide symporter.

The Tg and TPO promoters have been extensively studied, and multiple factors have been shown to be required for their expression (3, 4). To date, three transcription factors that specifically bind to and regulate these promoters have been cloned (2). The three transcription factors are as follows: thyroid transcription factor-1 (TTF-1), thyroid transcription factor-2 (TTF-2), and Pax8. TTF-1 (also named NKx 2.1 and T/EBP) is a homeodomain-containing protein expressed in embryonic diencephalon, thyroid, and lung (5). TTF-2 is a forkhead domain-containing protein expressed in pituitary and thyroid (6), and Pax8 is a member of the murine Pax family of paired domain-containing genes that is expressed in kidney, in the developing excretory system, and in the thyroid (7). We have focused our studies on the molecular mechanisms of action of TTF-1 and Pax8. These two transcription factors are present together only in the thyroid, suggesting that this unique combination could be responsible for early commitment and differentiation of thyrocytes. TTF-1 was originally identified as a protein binding to a DNA sequence that is present three times on both the Tg and the TPO promoters. Furthermore, TTF-1 was shown to be involved in the activation of thyroid-specific gene expression (8). In fact, TTF-1 is able to activate transcription from the Tg promoter and, albeit at a much lower level, from the TPO promoter both in thyroid and non-thyroid cells.

Parallel to its function in the thyroid, TTF-1 plays a critical role also in lung morphogenesis and in respiratory epithelial cell gene expression. It has been demonstrated that TTF-1 is required for the transcriptional activation of surfactant proteins A, B, and C (SP-A, SP-B, and SP-C) and Clara cell secretory protein (9–13). *ttf-1* gene inactivation in the mouse results in severe thyroid and lung hypoplasia (14), and the homozygous mice are not viable. Recently, it has been published that

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[1] The abbreviations used are: Tg, thyroglobulin; TPO, thyroperoxidase; SP, surfactant proteins A; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; DTT, dithiothreitol; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; LUC, luciferase.

TTF-1 and GATA-6 are co-expressed in the respiratory epithelium *in vivo* and in respiratory epithelial cells *in vitro*. It has been also demonstrated (15) that TTF-1 and GATA-6 directly interact and have a synergistic effect on the transcriptional activation of the SP-C promoter.

The Pax gene family encodes for DNA-binding proteins that are involved in the regulation of the development of a variety of tissues in different species. Specifically, Pax8 has been demonstrate to be required both for morphogenesis of the thyroid gland (16) and for maintenance of thyroid-differentiated phenotype (17). The molecular mechanisms involved in Pax8 control of differentiation have been investigated in detail. Pax8 binds to a single site on the Tg and on the TPO promoters, and in both cases, the Pax8-binding site overlaps with one of the TTF-1-binding site (4, 18). In addition, Pax8 was shown, in transient transfection assays, to activate transcription from the TPO and the Tg promoters in non-thyroid cells (18). Recently, direct evidence of the ability of Pax8 to activate transcription of thyroid-specific genes at their chromosomal locus was obtained (17). Interestingly, in Pax8 knockout mice the thyroid gland is barely visible and lacks follicular cells (16). Recent studies (19) have demonstrated that in rat differentiated thyroid cells in culture (the PC Cl3 cell line), the thyrotropin-stimulating hormone regulates the expression of both thyroglobulin and Pax8 genes by a cAMP-mediated mechanism.

Taken together, all the data present in the literature to date strongly suggest a fundamental role of Pax8 in the maintenance of functional differentiation in thyroid cells.

Despite the critical role during development and in human diseases of the Pax proteins, the biochemical basis of their function within the cell nucleus is poorly understood.

The functional role of TTF-1- and Pax8-binding sites within the Tg and TPO promoters has been studied in rat, bovine, and human Tg gene promoters (18, 20–22). Some studies (17) have suggested that there might be a functional cooperation between the two transcription factors in the transcriptional activation of thyroid-specific promoters. Recently, it has been proposed that TTF-1 and Pax8 cooperate in the stimulation of the TPO (23) and of the human Tg (22) gene promoters. However, the demonstration of a direct interaction has not yet been provided.

The aim of our study was to investigate a possible biochemical and functional interaction between TTF-1 and Pax8. Indeed, we were able to show a physical interaction between TTF-1 and Pax8, both in thyroid and non-thyroid cells. Co-immunoprecipitation experiments performed using thyroid cell extracts confirmed the existence of a complex between Pax8 and TTF-1 *in vivo*. Moreover, we observed an impressive synergistic effect of the two transcription factors on the Tg promoter activation. The synergism involves a specific region of the Tg promoter (region C), that is the most proximal to the start of transcription where TTF-1 and Pax8 binding sites overlap. In addition, by mutational analysis we have identified the portions of TTF-1 and Pax8 proteins responsible for the physical and functional interaction.

In conclusion, this paper describes a physical and functional interaction between an homeodomain-containing factor and a paired domain-containing factor, both involved in the regulation of tissue-specific transcription.

EXPERIMENTAL PROCEDURES

Plasmids—The GST-Pax8 fusion protein was generated by PCR amplification of Pax8 coding region and subsequent subcloning in the *Eco*RI site of the pGEX-4T vector. The orientation of the fragment and the correct frame of the fusion were verified by DNA sequencing.

3xFLAG-Pax8 was generated by PCR amplification of Pax8 coding region and subsequent cloning in the *Eco*RI-*Xba*I sites of the 3xFLAG CMV-10 vector (Sigma).

The plasmids used in transient transfection experiments have been described previously and are as follows: Tg-CAT (3); Tg-CAT Acore (24); TPO-LUC (4); CMV-TTF-1 (25); Δ1, Δ33, Δ2, Δ14, Δ3 (8); and pCMV5-Pax8 (18). Expression vectors encoding Pax8 splicing variants are described in Ref. 26.

The reporter construct Tg-CAT ABdel was generated by PCR amplification of a small portion of the Tg promoter (region C) and subsequent subcloning in *Sal*I-*Hin*dIII sites of the same reporter plasmid of Tg-CAT.

CMV-CAT and CMV-LUC plasmids were used as internal control in transfection assays. The DNA of all plasmids was prepared by Qiagen cartridges (Qiagen GmbH, Germany) and used for cell transfection.

Cell Culture and Transfection—PC Cl3 and HeLa cell lines have been described previously (27).

PC Cl3 cells were grown in Coon's modified F-12 medium (Seromed) supplemented with 5% calf serum (Invitrogen) and six hormones and growth factors as described by Ambesi-Impiombato and Coon (28).

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For transactivation experiments, cells were plated at a density of 3×10^5 cells/60-mm tissue culture dish, 5–8 h prior to transfection. Transfections were carried out with the FuGENE 6 reagent (Roche Diagnostics) as suggested by the manufacturer. The DNA/FuGENE ratio was 1:2 in all experiments.

Cells extracts were prepared after 48 h to determine either the levels of CAT protein with a CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) or LUC activities as described previously (6).

Transfection experiments were done in duplicate and repeated at least three times.

In Vitro Protein Interaction—GST-Pax8 protein was purified from BL21 (DE) LysS bacterial cells transformed with pGEX-Pax8. At A_{600} = 0.6, isopropyl-1-thio-β-D-galactopyranoside (0.1 mM final) was added to the culture to induce the expression of the fusion protein, and cells were harvested 4 h later. Cells were resuspended in Lysis Buffer (1× PBS, 0.5 mM EDTA, 1 mg/ml lysozyme, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors diluted 1:1000) and sonicated. 1% Triton X-100 was added, and the cell extract was centrifuged at 40,000 rpm for 40 min at 4 °C.

The supernatant was then subjected to affinity chromatography using glutathione-agarose beads (Amersham Biosciences). After binding, beads were washed three times with Washing Buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 5 mM DTT).

GST-Pax8 was eluted with a buffer containing 10 mM glutathione, 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, for 10 min at 4 °C. The eluted protein was store at −80 °C.

Protein concentration was judged from Coomassie Blue staining.

For the synthesis of bacterial TTF-1 protein, *Escherichia coli* cells were transformed with pQE12-TTF-1, an expression vector encoding for TTF-1 fused to a His_6 tag at the C terminus. The expression and the purification of TTF-1 protein were performed as described previously (46).

Pull-down assays were performed by challenging 4 μg of GST or GST-Pax8-purified proteins previously bound to glutathione-agarose beads with 3 mg of total protein extract prepared from PC Cl3 cells or HeLa cells. The binding reactions were carried out for 90 min at 4 °C on a rotating wheel, and then the beads were washed several times with a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and proteases inhibitors (from Sigma). The bound proteins were eluted by resuspending the beads directly in 2× SDS-PAGE sample buffer and heating at 95 °C for 3–5 min. before loading on the gel.

The co-immunoprecipitation experiment was performed by incubating 2 mg of total protein extract with 20 μl of anti-FLAG-agarose affinity gel (Sigma) overnight at 4 °C on a rotating wheel. The samples were then centrifuged, and the agarose gel-bound proteins were washed several times with a buffer containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and proteases inhibitors (from Sigma), resuspended in 2× SDS-PAGE sample buffer, and heated at 95 °C for 3–5 min before loading on the gel.

Indirect Immunofluorescence—Cells were grown directly on glass coverslips for 72 h, fixed in 3.7% formaldehyde in PBS for 20 min at room temperature, permeabilized for 7 min in 0.1% Triton X-100 in PBS, and incubated for 10 min in 0.1 M glycine in PBS. The coverslips were subsequently incubated for 1 h with a mixture of primary antibodies diluted in 0.5% bovine serum albumin in PBS and, after PBS washing, incubated for 20 min with a mixture of fluorescein-tagged goat anti-mouse and rhodamine-tagged goat anti-rabbit secondary antibodies diluted 1:50 in 0.5% bovine serum albumin in PBS. After final

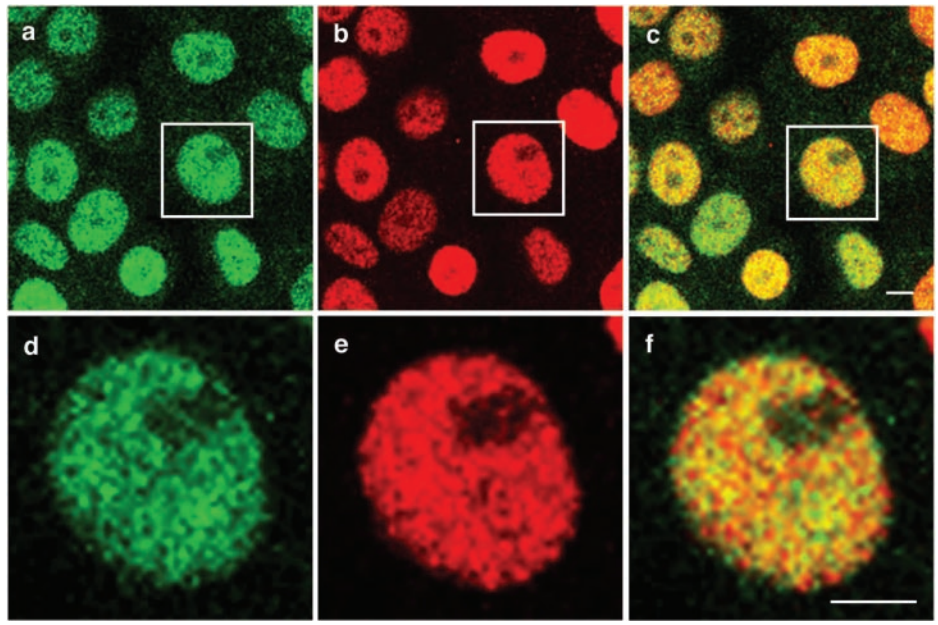

FIG. 1. **Co-localization by confocal microscopy of Pax8 and TTF-1 in PC cells.** PC cells were grown directly on glass coverslips and stained for immunofluorescence with the Ab-1 (clone 8G7G3/1) anti-TTF-1 monoclonal antibody (*a* and *d*), and the Ab187 anti-Pax8 polyclonal antibody (*b* and *e*). Fluorescein isothiocyanate and TRITC signals were acquired together at a confocal microscope, by line-wise scanning. The overlay of the two signals is shown in *c* and *f*. The nucleus within the *square* in *a–c* is shown at higher magnification in *d–f*, respectively. *Bar*, 5 μm

washings with PBS, the coverslips were mounted on a microscope slide using a 70% solution of glycerol in PBS.

Primary antibodies and sera were monoclonal anti-TTF-1 Ab-1 (clone 8G7G3/1) from Neo Markers (Lab Vision Corporation) and rabbit polyclonal anti-Pax8 Ab187 (29).

Confocal Scanning Laser Microscopy—Images were collected with a Zeiss LSM 510 confocal laser scanning microscope, equipped with a 488 nm argon ion laser, a 543 nm HeNe laser, and a Plan-Apochromat 63×/1.4 oil-immersion objective. Emitted fluorescence was detected using a BP 505–530 bandpass filter for fluorescein isothiocyanate, and a LP 560 long pass filter for TRITC. Pairs of images were collected simultaneously in the green and red channels. High magnification images were collected as 512 × 512 × 32 voxel images (sampling distance 110 nm lateral and 350 nm axial).

RESULTS

Pax8 and TTF-1 Co-localize in the Nucleus of Thyroid Cells—We have examined the pattern of distribution of Pax8 and TTF-1 in PC cells by immunolabeling and confocal microscopy. The nuclear localization of both transcription factors in thyroid cells in culture has been already shown by indirect immunofluorescence (19). The two antibodies that have been used, Ab-1 (clone 8G7G3/1) against TTF-1 and Ab187 against Pax8, are highly specific as indicated by immunoblot analysis (data not shown). Confocal microscopy examination of dual-labeled samples confirmed that the anti-TTF-1 (Fig. 1*a*) as well as the anti-Pax-8 (Fig. 1*b*) antibody stained only the nuclei, with the exclusion of nucleoli, and did not give any staining outside the nucleus. With either antibody the intensity of staining over individual nuclei varied significantly suggesting a different degree of expression of each transcription factor at the single cell level. At higher magnification (Fig. 1, *d* and *e*), it clearly appeared that both factors were not diffused within the nucleus but were instead localized in numerous interconnected nucleoplasmic domains, spread throughout the nucleus. The signals from the two immunostained transcription factors were acquired together, at high resolution, by line-wise scanning. It has been thus possible to determine that the patterns of distribution were quite similar and frequently superimposed, even though not identical, as inferred from the *yellow* output color (Fig. 1, *c* and *f*).

Pax8 and TTF-1 Interact Both in Thyroid and Non-thyroid Cells—In order to determine whether Pax8 and TTF-1 could physically associate, recombinant GST-Pax8 protein was purified from bacteria and used in pull-down experiments with total protein extracts prepared from thyroid and non-thyroid cells. In particular, we used the PC Cl3 cells as a source of thyroid-specific protein extracts and HeLa cells transiently transfected with an expression vector encoding for TTF-1 as a source of non-thyroid protein extracts containing exogenous TTF-1. Results of the binding reactions show that TTF-1 protein is specifically bound by GST-Pax8 but not by the unfused GST protein (Fig. 2*A*, compare *lanes 2* and *4* with *lanes 1* and *3*). In addition, the binding between Pax8 and TTF-1 occurs both in thyroid and non-thyroid cells (Fig. 2*A*, *lanes 2* and *4*), suggesting that the molecular basis of this interaction does not require a cell type-specific mechanism.

Note that TTF-1 in thyroid cells (PC Cl3 cell line, Fig. 2*A*, *lane 2*) appears as two bands, although when the cDNA is transiently transfected in non-thyroid cells (HeLa cell line, Fig. 2*A*, *lane 4*), only the higher molecular weight band is visible. The reason for this difference is still poorly understood.

To investigate the possibility that Pax8 and TTF-1 interact directly, pull-down experiments were performed using the fusion protein GST-Pax8 and bacterial TTF-1 protein affinity-purified. The glutathione-Sepharose beads loaded with GST-Pax8 were indeed able to co-precipitate the bacterial TTF-1 protein (Fig. 2*B*, *lane 3*), although the control reaction of beads loaded with the GST protein did not co-precipitate any protein (Fig. 2*B*, *lane 2*). These results demonstrated that the interaction between Pax8 and TTF-1, already observed in pull-down assays with total protein extracts, is indeed a direct protein-protein interaction.

Pax8 Forms a Protein Complex with TTF-1 in Vivo—To test whether the interaction observed *in vitro* in GST pull-down assays could be observed also *in vivo*, PC Cl3 thyroid cells were stably transfected with 3xFLAG-Pax8, an expression vector encoding for Pax8 fused to the FLAG epitope. Several independent clones were isolated, and the presence of 3xFLAG-Pax8 was determined by Western blot analysis using a specific anti-FLAG monoclonal antibody (data not shown). We identified 4 positive clones out of the 10 analyzed, and the neomycin-positive clones that did not express 3xFLAG-Pax8 were used as control of the subsequent experiments.

Anti-FLAG-agarose affinity gel was used to immunoprecipitate the 3xFLAG-Pax8 protein from total extracts prepared from clones 3xFLAG-P8-8 (positive clone) and 3xFLAG-P8-7

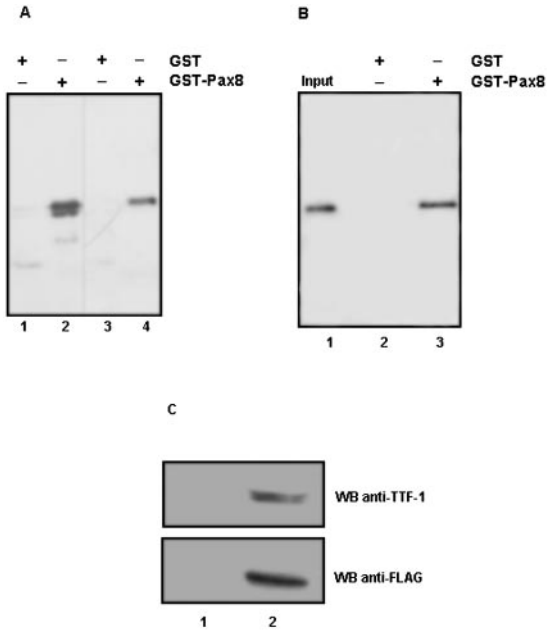

FIG. 2. **Pax8 and TTF-1 interact both *in vivo* and *in vitro*.** *A*, GST pull-down assay with GST-Pax8 immobilized on glutathione-Sepharose beads and protein extracts prepared from PC Cl3 or HeLa cells transiently transfected with the expression vector CMV-TTF-1. The extract of either cell types was incubated with GST or GST-Pax8. Both GST and GST-Pax8 beads were washed several times before being boiled, run on 10% SDS-polyacrylamide gel, and analyzed by Western blot using a TTF-1-specific antibody. *Lanes 1* and *2*, PC Cl3 protein extract. *Lanes 3* and *4*, protein extract of HeLa cells transfected with CMV-TTF-1. *B*, full-length purified bacterial TTF-1 was generated as described under "Experimental Procedures" and incubated with GST-Pax8 and GST control, separately. Both GST and GST-Pax8 beads were washed several times before being boiled, run on 10% SDS-polyacrylamide gel, and analyzed by Western blot using a TTF-1-specific antibody. *Lane 1*, purified bacterial TTF-1. *Lane 2*, TTF-1 protein precipitated with GST for control. *Lane 3*, TTF-1 protein precipitated with GST-Pax8. *C*, for the co-immunoprecipitation experiment 2 mg of total protein extract were incubated with anti-FLAG-agarose affinity gel. The bound proteins were separated on 10% SDS-PAGE and analyzed by Western blot using first a TTF-1-specific polyclonal antibody and subsequently a monoclonal anti-FLAG antibody (Sigma). *Lane 1*, protein extract of clone 3xFLAG-P8-7. *Lane 2*, protein extract of clone 3xFLAG-P8-8.

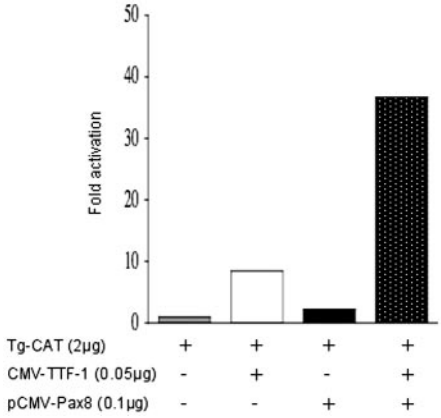

FIG. 3. **Synergistic activation of the thyroglobulin promoter by Pax8 and TTF-1.** HeLa cells were transiently transfected with the reporter plasmid Tg-CAT containing the minimal promoter region of the rat thyroglobulin gene, with or without the expression vectors encoding for TTF-1 and Pax8. The cells were subsequently harvested and assayed for CAT activity. Folds of activation are considered as ratio between values obtained with and without co-transfection of the expression vectors. CMV-LUC was added as internal reference, and CAT values were normalized to the LUC activity. Values are the mean of at least three independent experiments.

(negative clone). Subsequently, the bound proteins were subjected to Western blot analysis after separation by SDS-PAGE. Western blot developed with a specific anti-TTF-1 polyclonal antibody showed the presence of TTF-1 co-immunoprecipitated protein only in the extract prepared from clone 3xFLAG-P8-8 (Fig. 2*C*).

Pax8 and TTF-1 Synergistically Stimulate Transcription from the Tg Promoter—It was reported previously that in transient transfection assays in HeLa cells, TTF-1 is able to activate transcription from a reporter construct in which the Tg minimal promoter is subcloned upstream of the CAT gene (Tg-CAT (8)). In contrast, in the same experimental model system Pax8 is a quite poor activator of the Tg promoter (18). However, several lines of evidence indicate that Pax8 is a transcription factor strictly required for Tg gene transcription in thyroid cells (17, 19, 20). To test whether Pax8 and TTF-1 could synergistically stimulate transcription from thyroid-specific gene promoters, expression vectors encoding for Pax8 and TTF-1 were co-transfected in HeLa cells together with the reporter construct Tg-CAT, separately or in combination. The two transcription factors when transfected separately enhanced transcription from the Tg promoter, albeit to a different extent as expected (Fig. 3). Interestingly, co-expression of TTF-1 and Pax8 led to a marked synergism in the activation of the Tg promoter (Fig. 3). Moreover, a detailed analysis of the synergistic effect demonstrated that for the functional interaction between Pax8 and TTF-1 on the Tg promoter the ratio of the two transcription factors is crucial. It is worth mentioning that when a very small DNA concentration of both transactivators are used, it is possible to observe the synergism. In contrast, higher DNA concentrations still have the ability of activating transcription from the promoter but do not show the synergism any longer (data not shown). A clear dose-dependent effect was observed as reported in other studies (23).

TTF-1 N-terminal Domain Is Required for the Synergy with Pax8—Previous studies demonstrated that TTF-1 activates transcription via a functional redundancy of the N-terminal and C-terminal domains (8). To determine which of the two domains mediates the synergy with Pax8 in the transcriptional activation of the Tg promoter, we have used deletion mutants described previously (8). The mutants, named Δ14 and Δ3, were transfected in HeLa cells together with the reporter construct Tg-CAT, in the absence or presence of Pax8. As expected, both mutants were able to activate the Tg promoter as the wild-type TTF-1 molecule (Fig. 4*A*). However, we demonstrated that mutant Δ14 that contains the N-terminal activation domain and the homeodomain is able to synergize with Pax8 on Tg-CAT-like wild-type TTF-1 (Fig. 4*A*). On the contrary mutant Δ3, which contains the homeodomain and the C-terminal activation domain, lacks the ability to exert the synergism with Pax8 (Fig. 4*A*). Therefore, we conclude that even though in previous studies it was suggested that the two activation domains of TTF-1 were functionally equivalent, only the N-terminal domain of TTF-1 is able to synergize with Pax8. To map further TTF-1 N-terminal sequences involved in transcriptional activation with Pax8, we have tested additional TTF-1 N-terminal deletion mutants described previously (8), such as Δ1, Δ2, and Δ33 (Fig. 4*B*). As before, the mutants were transfected in HeLa cells together with the reporter construct Tg-CAT, in the absence or in the presence of Pax8. The results obtained for each of the mutants tested indicate that the sequence downstream from amino acid 51 in TTF-1 N-terminal domain is required for the synergistic activation with Pax8. In fact, deletion mutant Δ1 behaves as wild-type TTF-1 (Fig. 4*B*), suggesting that the region spanning between amino acids 1 and 51 is not essential for the synergy with Pax8. At the same time,

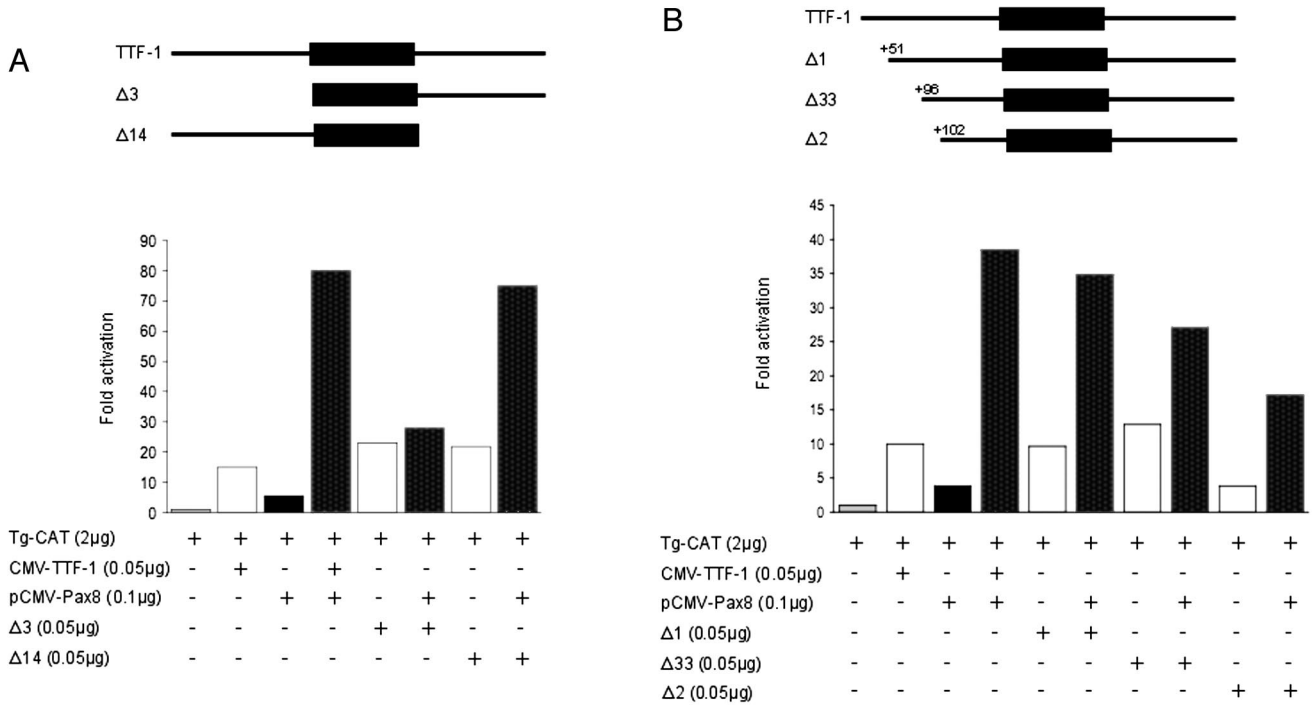

FIG. 4. **The synergistic effect on the thyroglobulin promoter requires TTF-1 N-terminal domain.** *A*, schematic representation of the different TTF-1 proteins used in transactivation experiments. Δ3 contains the homeodomain and the C-terminal activation domain. Δ14 contains the N-terminal activation domain and the homeodomain. HeLa cells were transiently transfected with the reporter plasmid Tg-CAT and the expression vectors encoding either wild-type TTF-1 or the deletion mutants. Folds of activation are considered as ratio between values obtained with and without co-transfection of the expression vectors. CMV-LUC was added as internal reference, and CAT values were normalized to the LUC activity. Values are mean of at least three independent experiments. *B*, schematic representation of TTF-1 N-terminal mutants. HeLa cells were transfected as described before. Mutants Δ1, Δ33, and Δ2 lack amino acids between 1 and 51, 1 and 92, and 1 and 102, respectively. CMV-LUC was added as internal reference, and CAT values were normalized to the LUC activity. Values are mean of at least three independent experiments.

mutants Δ33 and Δ2 show a progressively reduced ability in the cooperation with Pax8 (Fig. 4*B*), thus indicating that the portion of the protein downstream from residue 51 is involved in the synergism.

In order to test whether the different ability to synergize with Pax8 of mutants Δ14 and Δ3 was due to a different ability in establishing a protein-protein interaction, we have performed pull-down experiments with protein extracts prepared from HeLa cells transiently transfected with the expression vectors encoding for Δ14 and Δ3 mutants. Results of the binding reactions show that Δ14 protein is specifically bound by GST-Pax8, although Δ3 protein is not, thus demonstrating that the biochemical interaction is required for the synergism (Fig. 5).

Identification of Pax8 Domains Required for the Functional Cooperation—There are several reported isoforms of Pax8 protein (26, 31). Among those, Pax8a is the full-length isoform that contains all 10 exons and encodes for the most abundant Pax8 protein species. Pax8b does not contain exon 8, although Pax8c lacks exons 7 and 8. In this latter isoform, as a consequence of the splicing of two exons, the shift in the reading frame generates a proline-rich C terminus and a premature termination of translation (Fig. 6*A*).

The C-terminal part of Pax8 full-length protein is rich in proline, serine, and threonine (PST domain) and functions as a transcriptional activation domain (32). In addition, data obtained studying various Pax genes showed that the above regulatory module present in the C-terminal region has been conserved among the members of the family (32).

The different Pax8 isoforms encode for proteins that differ in the C terminus and therefore could differ, as suggested in previous studies, in their transactivation properties (26). In all our experiments we have used the full-length Pax8 cDNA,

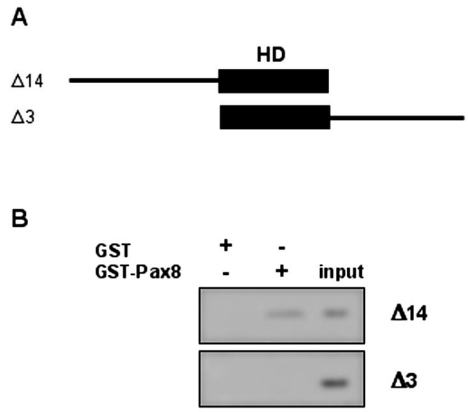

FIG. 5. **The interaction with Pax8 requires TTF-1 N-terminal domain.** *A*, schematic representation of TTF-1 mutated proteins. Δ3 contains the homeodomain and the C-terminal activation domain. Δ14 contains the N-terminal activation domain and the homeodomain. *B*, GST pull-down assay with GST-Pax8 immobilized on glutathione-Sepharose beads and protein extracts prepared from HeLa cells transiently transfected with the expression vector encoding for Δ14 and Δ3 proteins. The extracts were incubated with GST or GST-Pax8. Both GST and GST-Pax8 beads were washed several times before being boiled, run on 10% SDS-polyacrylamide gel, and analyzed by Western blot using a TTF-1-specific antibody (for Δ14) or an antibody (mAb 9E10 from Santa Cruz Biotechnology, Inc.) recognizing the Myc tag epitope (for Δ3).

namely the Pax8a isoform. We then asked whether the C terminus of the protein could be the portion involved in the synergism between Pax8 and TTF-1 in the transcriptional activation of the Tg promoter. To answer this question, we tested whether Pax8a, Pax8b, and Pax8c behave differently with re-

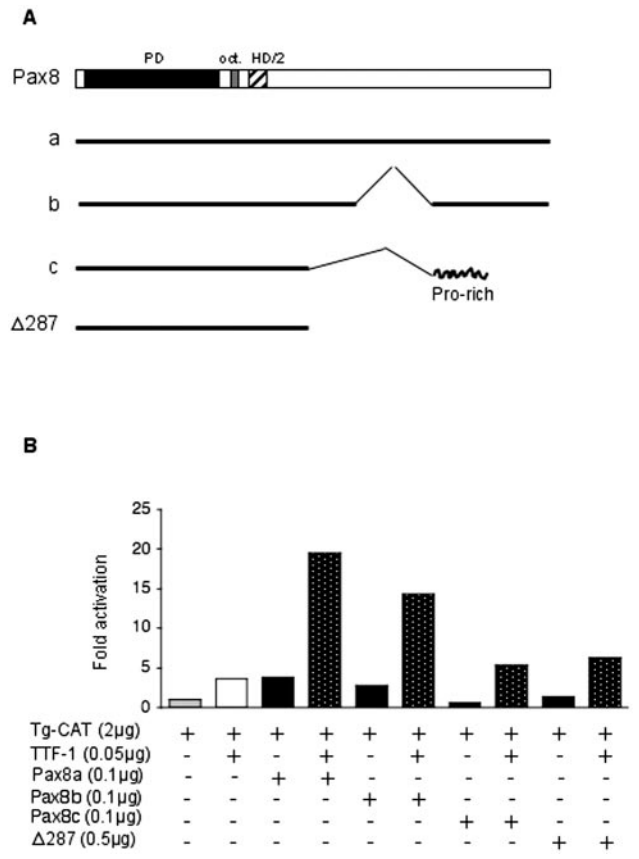

FIG. 6. **Pax8 C-terminal domain is involved in the cooperation with TTF-1.** *A*, schematic drawing of Pax8 isoforms. Pax8a is the full-length isoform. Pax8b lacks exon 8, although Pax8c lacks exon 7 and 8. The proline-rich domain arising due to the alternative splicing results in the reading frameshift and premature termination of translation. Mutant Δ287 lacks the C-terminal domain downstream exon 6. *PD*, paired domain; *oct*, octapeptide; *HD/2* partial homeodomain. *B*, HeLa cells were transiently transfected with the reporter plasmid Tg-CAT, the expression vector encoding TTF-1, and the different Pax8 isoforms or deletion mutant. Folds of activation are considered as ratio between values obtained with and without co-transfection of the expression vectors. CMV-LUC was added as internal reference, and CAT values were normalized to the LUC activity. Values are mean of at least three independent experiments. Pax8a and Pax8b synergistically activate Tg promoter with TTF-1. Pax8c and Δ287 are not able to cooperate with TTF-1.

spect to the synergism with TTF-1 in Tg activation in HeLa cells.

It was demonstrated previously (17) that the isoforms Pax8a, -b, and -c are equally able to bind the same sequence derived from the Tg promoter. Thus, we co-transfected expression vectors encoding for Pax8a, Pax8b, and Pax8c, in the presence or absence of TTF-1, together with the Tg promoter reporter construct in HeLa cells. The results shown in Fig. 6*B* demonstrate that although Pax8a and Pax8b are equally capable of functionally cooperating with TTF-1, the isoform Pax8c is no longer able to do so. Accordingly, a deletion mutant, Δ287, lacking the entire C-terminal region, is also not able to show any synergism when tested in the same experimental conditions (Fig. 6*B*). These results clearly demonstrate that the C terminus of the protein is the portion of Pax8 involved in the synergism with TTF-1 that we observed on the Tg promoter.

The TTF-1 and Pax8 Overlapping Binding Sites Are Involved in the Synergism—Pax8 binds to a single site on the Tg promoter, and the binding site overlaps with one of the TTF-1-binding sites (18). To elucidate the molecular mechanism taking place in the synergism between the two transcription factors, we asked which of the three TTF-1-binding sites present on the Tg promoter was involved in the observed effect. Thus, we investigated the effects of Pax8 and TTF-1 on reporter constructs containing point mutations of the Tg promoter. We first tested the Tg-CAT Acore mutant, which contains point mutations that abolish the binding of TTF-1 at its distal site on the promoter (site A (24)). Therefore, we co-transfected the Tg-CAT Acore promoter mutant with both TTF-1 and Pax8 in the same experimental conditions used for the wild-type promoter. Interestingly, we were able to observe a strong synergism between TTF-1 and Pax8 in the transcriptional activation of the promoter (Fig. 7). The extent of the synergism on Tg-CAT Acore was comparable with that observed on the wild-type Tg promoter. This result strongly suggest that the molecular mechanisms at the basis of the synergistic effect lie within the so-called region C of the Tg promoter (3, 18), which contains the TTF-1-binding site proximal to the start of transcription overlapping the Pax8-binding site. To test this hypothesis, we have used the Tg-CAT Ccore mutant, which contains point mutations that abolish the binding to region C of the promoter (Fig. 7). When we tested this mutated promoter in co-transfection experiments in HeLa cells, we did not observe any synergy. Thus, our results point out at a molecular mechanism involving specifically site C of the Tg promoter.

Pax8 and TTF-1 Do Not Strongly Synergize on the TPO Promoter—Previous studies (4) have shown that in HeLa cells Pax8 strongly activates transcription from the TPO promoter. In order to investigate whether the functional interaction between Pax8 and TTF-1 occurs on another thyroid-specific promoter structurally very similar to the Tg one, HeLa cells were transfected with Pax8 and TTF-1 and the reporter construct TPO-LUC (4). As expected Pax8 activates transcription from the TPO promoter severalfold, whereas TTF-1 is less efficient (Fig. 8). However, when co-transfected together, Pax8 and TTF-1 are unable to synergize on the TPO promoter (Fig. 8). These results indicate that although both transcription factors bind to the Tg and TPO promoters in regions displaying extensive sequence homology, their functional interaction is promoter-specific.

DISCUSSION

In this study we show that the paired domain-containing transcription factor Pax8 and the homeodomain-containing transcription factor TTF-1 co-localize in the nucleus of thyroid cells and synergistically activate transcription from the promoter of thyroglobulin, a thyroid-specific gene. A direct biochemical interaction of the two factors was observed both in *in vitro* and *in vivo* assays. This evidence, together with their well established role during development/morphogenesis of the thyroid gland, supports the hypothesis that a protein-protein interaction between these two transcription factors may play a relevant role in the expression of target genes in the thyroid. Recently, *pax8* was shown to be a master gene for the maintenance of thyroid-differentiated phenotype, being important for the transcriptional activation of all the differentiation markers such as thyroglobulin, thyroperoxidase, and sodium/iodide symporter genes (17). Pax genes encode evolutionary conserved transcription factors that act high up in the regulatory hierarchy controlling the development of various organs. From the analysis of the transgenic or knockout mice, it has become clear that *pax* genes are key regulators during crucial steps of the organogenesis of various tissues (33–36). In particular, Pax8 knockout mice have a barely visible thyroid gland, which is deprived of the follicular cells (16), and human patients suffering from congenital hypothyroidism have been shown to carry mutations in the *Pax8* gene (37–39). TTF-1 is also an important factor for thyroid morphogenesis. TTF-1 knockout mice lack the thyroid and the pituitary gland and display severe defects in

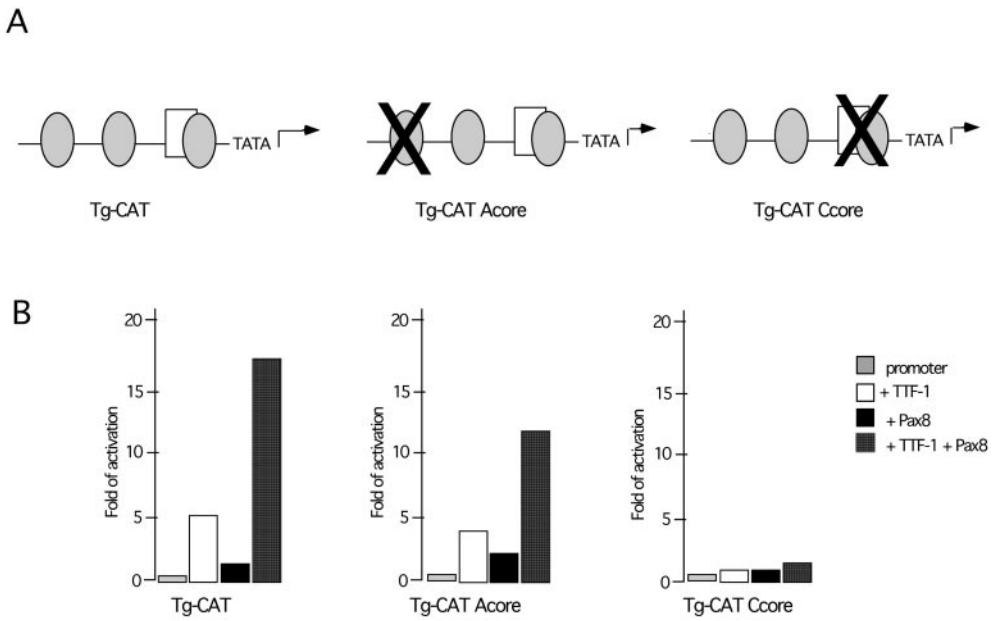

FIG. 7. **Mapping of the cis-elements involved in Pax8/TTF-1 synergy.** *A*, schematic representation of the Tg reporter constructs: wild-type (*Tg-CAT*), mutated in the distal TTF-1-binding site (*Tg-CAT Acore*) and mutated in the TATA box proximal Pax8 and TTF-1-binding sites (*Tg-CAT Ccore*). *Gray ovals* represent TTF-1, and the *white rectangle* represents Pax8. *B*, HeLa cells were transiently transfected with the reporter plasmids containing the wild-type Tg promoter (*Tg-CAT*), or with the mutated Tg-CAT Acore and Tg-CAT Ccore promoters with or without the expression vectors encoding for TTF-1 and Pax8. CMV-LUC was added as internal reference, and CAT values were normalized to the LUC activity. Folds of activation are considered as ratio between values obtained with and without co-transfection of the expression vectors. Values are mean of at least three independent experiments.

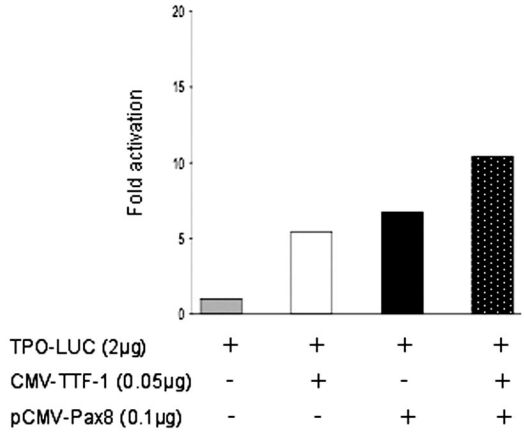

FIG. 8. **Pax8 and TTF-1 do not synergize on the TPO minimal promoter.** HeLa cells were transiently transfected with the reporter plasmid TPO-LUC containing the minimal promoter region of the rat thyroperoxidase gene, with or without the expression vectors encoding for TTF-1 and Pax8. The cells were subsequently harvested and assayed for LUC activity. Folds of activation are considered as ratio between values obtained with and without co-transfection of the expression vectors. CMV-CAT was added as internal reference, and LUC values were normalized to the CAT activity. Values are mean of at least three independent experiments.

the ventral area of the forebrain and in peripheral lung parenchyma (14).

In the present study, we show that Pax8 and TTF-1 are able to interact directly *in vitro* in pull-down experiments, both in thyroid and non thyroid cell extracts. In addition, by co-immunoprecipitation we also show that Pax8 and TTF-1 form a complex *in vivo*. Our data provide the first strong evidence of the existence of a biochemical interaction between these two transcriptional activators. Moreover, we clearly show that the presence of both factors together exclusively in thyroid cells has indeed a functional relevance. In fact, their synergistic effect in the transcriptional activation of the Tg promoter represents an example of how tissue-specific gene expression may be achieved.

Previous results from our laboratory already suggested that TTF-1 and Pax8 might cooperate in the transcriptional activation of Tg gene expression in thyroid cells (17). Furthermore, it was recently claimed that Pax8 and TTF-1 might cooperate in the regulation of thyroid-specific gene expression (22, 23). However, none of above-mentioned studies unravel a protein-protein interaction between Pax8 and TTF-1. Interestingly, our previous data showed (18) that the binding site of Pax8 on the Tg promoter overlaps one of the TTF-1-binding sites and that, at least *in vitro*, the two factors cannot bind together to the same DNA region. The results presented in this article provide the evidence of a direct interaction between Pax8 and TTF-1, which occurs via protein-protein interaction in the absence of DNA. It is likely that the different experimental assays used in the previous study are the reason of our failure to detect the interaction.

What could be the mechanism leading to the synergistic activation of the Tg promoter by TTF-1 and Pax8 transcription factors? Interactions among transcription factors that bind to separate promoter elements may depend on various events, such as distortion of DNA structure. We have now established that the molecular mechanisms leading to the synergistic activity of Pax8 and TTF-1 implicate region C of the Tg promoter (3, 18), which is where the binding sites of the two factors overlap. It is, however, still unclear if the two proteins are both binding to the DNA or if only one of them is binding and thus recruits the other one to the promoter. Further investigation is required to elucidate which protein is indeed bound to the DNA *in vivo*. By mutational analysis we have determined the regions of Pax8 and TTF-1 proteins that are involved in the functional cooperation. TTF-1 contains two distinct activation domains, one at the N terminus and the other at the C terminus, which have been shown to be functionally equivalent (8). We demonstrated that only one domain, the N-terminal domain, is able to mediate the synergism with Pax8. In addition, we show that

the synergy requires a biochemical interaction and that TTF-1 N-terminal domain is involved in such an interaction. At the same time we have also established that the C-terminal portion of Pax8 protein is necessary for the cooperative effect. In this part of the protein that is very rich in proline, serine, and threonine lies the activation domain of Pax8.

Moreover, it has become evident that, despite the high similarity of the Tg and TPO promoter architecture, the molecular mechanisms leading to transcriptional activation are different. In contrast with the transactivation synergy observed on the Tg promoter, no such effect was observed on the TPO promoter. Recently, Miccadei *et al.* (23) observed a synergistic activity of TTF-1 and Pax8 that relies on the TPO promoter/enhancer interplay. In their study they show that the synergistic activity can be observed only in the presence of the enhancer element. In our experiments we have used the minimal region of the TPO promoter that is able to drive thyroid-specific gene expression (4), and on this regulatory element we are not able to show any synergism likely because of the lack of the upstream enhancer. It is possible that Pax8 and TTF-1 interact in complex ways at cis-active elements of target genes.

There is increasing evidence that TTF-1 functions cooperatively with a number of the other transcription factors, forming complexes on regulatory regions of target genes. TTF-1 interacts with retinoic acid receptors and co-factors (40), AP-1 family members (41), BR22 (42), CBP/p300 (43), TGD (T:G mismatch-specific thymine DNA glycosylase) (44), and GATA-6 (15). Pax8, as it has already been observed for other members of the *pax* gene family (30, 45), could play a role in the recruitment to the Tg promoter of other factors, like TTF-1, and one or more co-activators. Our current hypothesis is that, depending on the promoter context within target genes, TTF-1 and Pax8 can act separately or in cooperation with various factors or co-factors to regulate gene expression. Thus, these different classes of transcriptional regulatory molecules may form a network through protein-protein and protein-DNA interactions in thyroid follicular cells, allowing the fine-tuning of thyroid-specific gene expression.

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The Paired Domain-containing Factor Pax8 and the Homeodomain-containing Factor TTF-1 Directly Interact and Synergistically Activate Transcription

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