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The Thyroid Transcription Factor 2 (TTF-2) Is a Promoter-Specific DNA-Binding Independent Transcriptional Repressor

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The thyroid transcription factor TTF-2 is a forkhead-containing protein involved in thyroid-specific gene expression and necessary for thyroid morphogenesis. In this paper, we demonstrate that TTF-2 is able to inhibit the activity of the thyroid-specific transcription factors TTF-1 and Pax-8 only on certain promoters. We identified the minimal protein domain responsible for repressor activity, which behaves as an independent functional domain, and we show that repression by TTF-2 is DNA-binding independent. We suggest that TTF-2 is able to interfere with a specific cofactor required for TTF-1 and Pax-8 activity. © 2000 Academic Press

Key Words: **transcription; repression; thyroid; TTF-1; TTF-2; Pax8.**

TTF-2 is a forkhead-containing protein (1) highly enriched in thyroid follicular cells (2, 3). TTF-2 was discovered as a thyroid-specific DNA binding activity recognizing the promoter of thyroglobulin (Tg) and thyroidperoxidase (TPO) genes. Subsequent studies suggested that TTF-2 might be responsible of mediating hormonal control of Tg and TPO gene expression (4) and that expression of the gene encoding TTF-2, denominated titf2, is itself under hormonal regulation (1). However, disruption of the titf2 gene in mice demonstrated that TTF-2 plays an important role in thyroid morphogenesis at stages preceding Tg and TPO gene expression (5), consistent with the onset of titf2 expression, that begins at day 9 of mouse development (1). Thus, it appears as if TTF-2 controls the expression of different functions, and hence of different transcriptional units, during development of the thyroid follicular cells. Such a complex function is not unusual for members of the forkhead family of DNA binding proteins, as several member of this family has been strongly implicated in developmental regulation (6). For example, it has been demonstrated that the HNF-3 proteins are critical factors in maintaining the potential for proper differentiation of specific tissues (7, 8). However, some of them show a distinct spatial patterns of expression during embryogenesis (9), suggesting that they play a role not only in tissue specific gene expression, but also in the commitment of defined cell lineage. In agreement with this hypothesis, it has been demonstrated that HNF3-β is involved both in the gastrulation process and liver specific gene expression (10, 11).

The multiple role played by TTF-2 during development and differentiation of thyroid cells prompted us to study the transcriptionally active domain of this protein, leading to the discovery that TTF-2 can function as a transcriptional repressor (1).

In the present study we confirm that TTF-2 is a promoter-specific transcriptional repressor, further expanding on its promoter specificity, on its DNA-binding independence and on the ability to interfere only with some transcriptional activation domains. We also precisely define the TTF-2 repression domain and show that it is located at the carboxy terminus of the TTF-2 protein. Taken together, these data suggest that TTF-2 may act as repressor, perhaps by interacting with cofactor(s) involved in the activation of thyroid specific gene expression and provide structural information that could be essential for their identification.

MATERIALS AND METHODS

Cell culture and transfection. HeLa cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum.

Abbreviations used: NIS, sodium/iodide symporter; DBD, DNA binding domain.

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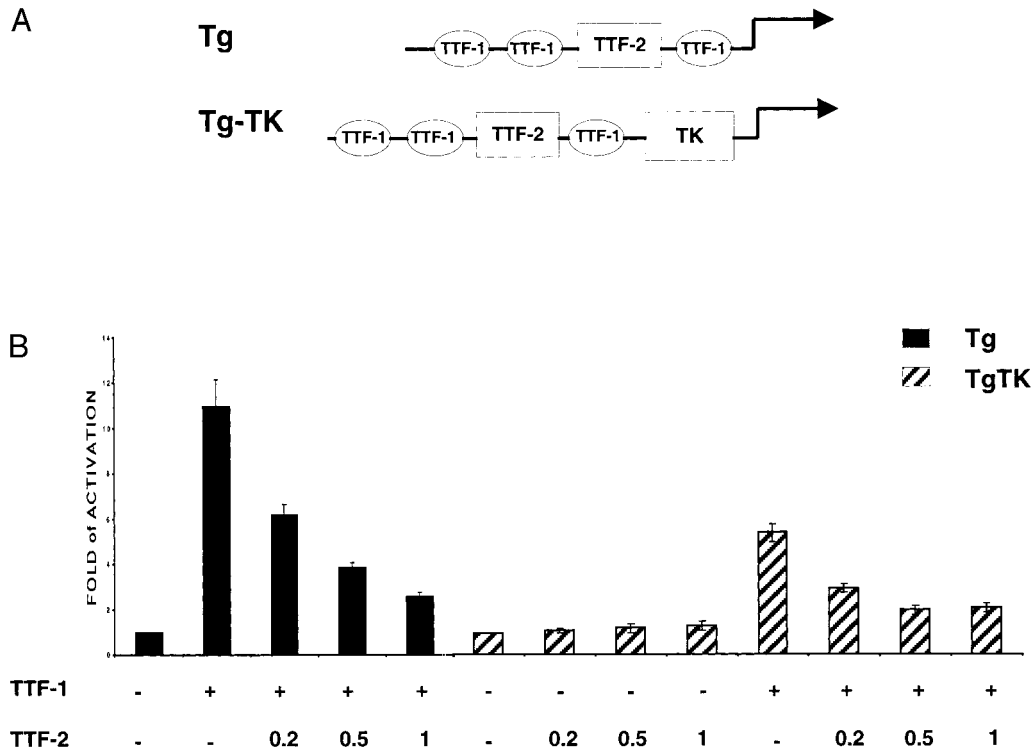

FIG. 1. Effect of TTF-2 cotransfection on the activity of Tg and TgTK. (A) Structure of the reporter constructs with the localization of the transcription factors binding sites. (B) HeLa cells were transiently transfected with the reporter plasmids (5 μg), with TTF-1 (0.1 μg), and with increasing amount of TTF-2 (expressed as μg of DNA) as indicated. CMVLUC was cotransfected to normalize for transfection efficiency. The bars show reporter activity and are expressed as fold of activation above that observed in the absence of TTF-1 expression (taken as 1). Black bars represent Tg activity and striped bars indicate TgTK activity. Values represent the average of three independent experiments.

For transient expression assay, cells were plated at 5×10^5 cells/60 mm tissue culture dish 4–6 h prior to transfection. Transfections and measurement of luciferase activity on cell extracts were determined as described. CAT expression was detected using an enzyme immunoassay (CAT ELISA, Boehringer-Mannheim) according to the manufacturer's instructions.

Plasmids. The deletion mutants of the C-terminal domain of TTF-2 have been obtained by polymerase chain reaction using primers encoding the amino acids at the ends of each fragment; all deletion were cloned replacing the full-length DNA of CMV-TTF-2 plasmid (1). To construct the chimeric genes encoding the LEXA DBD fused with the C-terminal domain of TTF-2, we obtained the C-terminal mutant by polymerase chain reaction, using primers encoding the amino acids at the ends of each fragment; the deletion was cloned in frame at the C-terminal of LEXA DBD. The reporter TgTK plasmid was constructed cloning the fragment *Sal*I–*Nhe*I upstream the TK minimal promoter (12).

The plasmids CMVTTF-1, pTACAT3, TPO-CAT, CMVLUC, C5E1b-CAT, pNISTKLUC3, CMVPax8 has been described (13, 14).

Band shift assay. Cellular extracts were prepared as described (1). The binding reaction was carried out in a buffer containing 40 mM Hepes, pH 7.9, 45 mM KCl, 1 mM dithiothreitol and 0.3 mg/ml poly(dI:dC). After 30 min of incubation, free DNA and DNA-protein complexes were resolved on a 10% polyacrylamide gel run in 0.5× TBE (2 mM EDTA, 90 mM boric acid, 90 mM Tris–HCl, pH 8.0) at 4°C. The gel was dried and then exposed to an X-ray film at −80°C. Oligonucleotide K, used to measure TTF-2 deletion mutants binding activity, has been described (1).

RESULTS

TTF-2 is a promoter-specific transcriptional repressor. By transient transfection experiments we have previously shown that TTF-2 is able to inhibit TTF-1 transcriptional activity (1). Interestingly, TTF-2 does not inhibit TTF-1 activity on C5E1b, an artificial promoter containing 5 DNA binding sites for TTF-1 (1), thus suggesting that the repression activity of TTF-2 is promoter specific. This observation led us to further investigate the promoter specificity of TTF-2 repression activity. As shown in Fig. 1B, the Tg promoter (pTACAT3) is efficiently activated by TTF-1 in HeLa cells and this activation is abolished by TTF-2 in a dose-dependent manner. A similar repression is observed on the Tg-TK promoter, a reporter construct containing the Tg regulatory region upstream of the TK minimal promoter (Fig. 1A and (12)). However, no effect is detectable on the basal activity of this construct in the absence of TTF-1 (Fig. 1B), thus demonstrating that TTF-2 interferes only with TTF-1-dependent activation.

We further investigated the specificity of TTF-2 repression activity on Pax8 transcriptional activity. We used both the TPO promoter and the NIS enhancer,

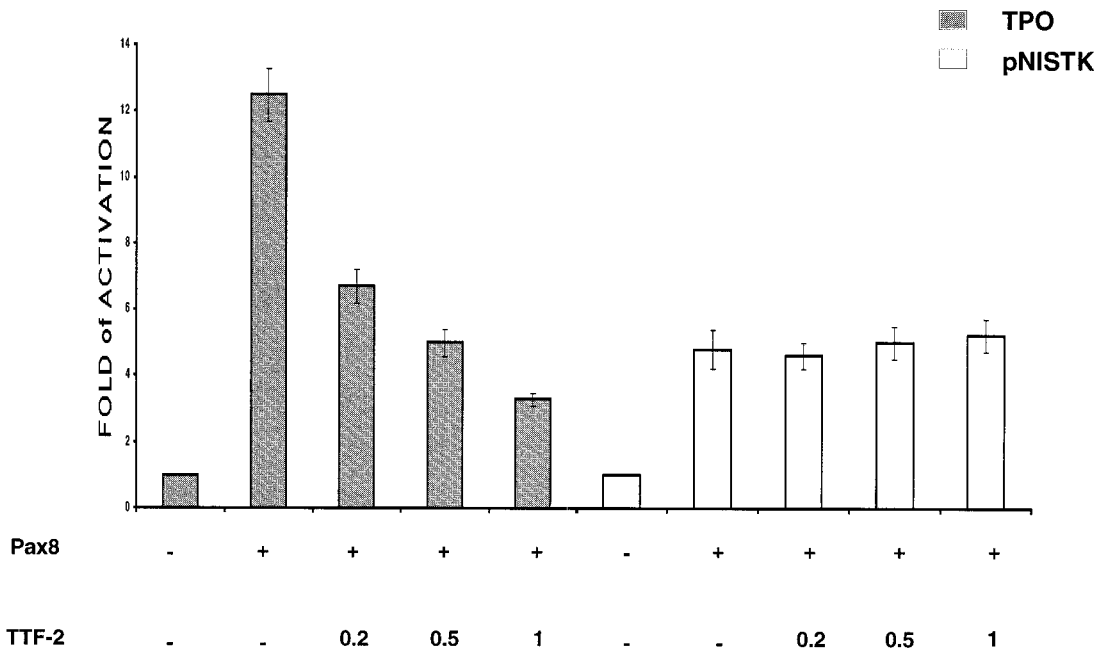

FIG. 2. Effect of TTF-2 cotransfection on Pax8-mediated activity of TPO promoter and NIS enhancer. HeLa cells were transiently transfected with the reporter plasmids (5 μg), with Pax 8 (0.1 μg) and with increasing amount of TTF-2 (expressed as μg of DNA) as indicated. CMVLUC was cotransfected to normalize for transfection efficiency for TPOCAT transfections. CMVCAT was used to normalize the transfections with pNISTKLUC. Gray bars show TPO activity and empty bars indicate NIS activity. Values are expressed as fold of activation above that observed in the absence of Pax8 expression (taken as 1) and represent the average of three independent experiments.

since it has been demonstrated that both can be activated by coexpression of Pax8 in nonthyroid cells (Fig. 2). Interestingly, TTF-2 represses Pax8 activity on TPO promoter, while Pax8-mediated activation of NIS enhancer is not affected by TTF-2. Thus, these data confirm that TTF-2 is a transcriptional repressor promoter specific, able to recognize different promoter architecture.

TTF-2 inhibition is activation domain specific. We next investigated the ability of TTF-2 to interfere with the activity of distinct activation domain. To this end we used TTF-1 deletion mutants Δ14 and Δ3 (Fig. 3A and (13)) and we analyzed their activity on the TgCAT reporter gene (pTACAT3) both in the absence and in the presence of TTF-2. Both deletion mutants contain the homeodomain of TTF-1 and thus are able to bind the Tg promoter and to induce transcription of the TgCAT reporter gene (13). As shown in Fig. 3B, TTF-2 interferes with the activity of the Δ3 mutant, while it has no effect on the transcription induced by the Δ14 mutant, strongly suggesting that the two activation domains of TTF-1 activate transcription with different mechanisms, only one of which is interfered by TTF-2.

Localization of the TTF-2 repression domain. To localize the domain required for the repressor activity of TTF-2, deletion mutants of C-terminus were tested, in transient transfection experiments, for the ability to inhibit TTF-1 activation of the TgCAT reporter gene (pTACAT3). To control for the level of expression of each mutant, the DNA binding activity of the mutant proteins was examined in transfected cells by gel mobility shift assay, using the oligonucleotide K (1) specifically recognized by TTF-2 (Fig. 4A). The results of

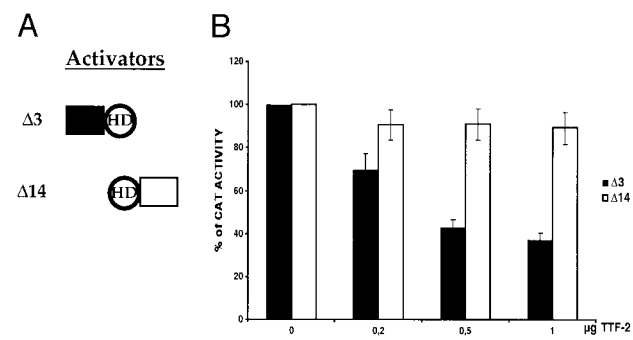

FIG. 3. TTF-2 inhibits specific activation domains. (A) Structure of the activation domains: HD indicates the homeodomain of TTF-1. Δ3 contains the entire N-terminal domain and the HD of TTF-1. Δ14 contains the HD and the entire C-terminal domain of TTF-1. (B) HeLa cells were transiently transfected with TgCAT (5 μg), with TTF-1 deletion mutants containing either N-terminal (0.2 μg) or C-terminal (0.2 μg) activation domain and with increasing amount of TTF-2 (expressed as μg of DNA) as indicated. CMVluc was cotransfected to normalize for transfection efficiency. Black bars represent Δ3 transcriptional activity; empty bars show Δ14 activity. Values are expressed as percentage of activity above that observed in absence of TTF-2 expression (taken as 100%) and represent the average of three independent experiments.

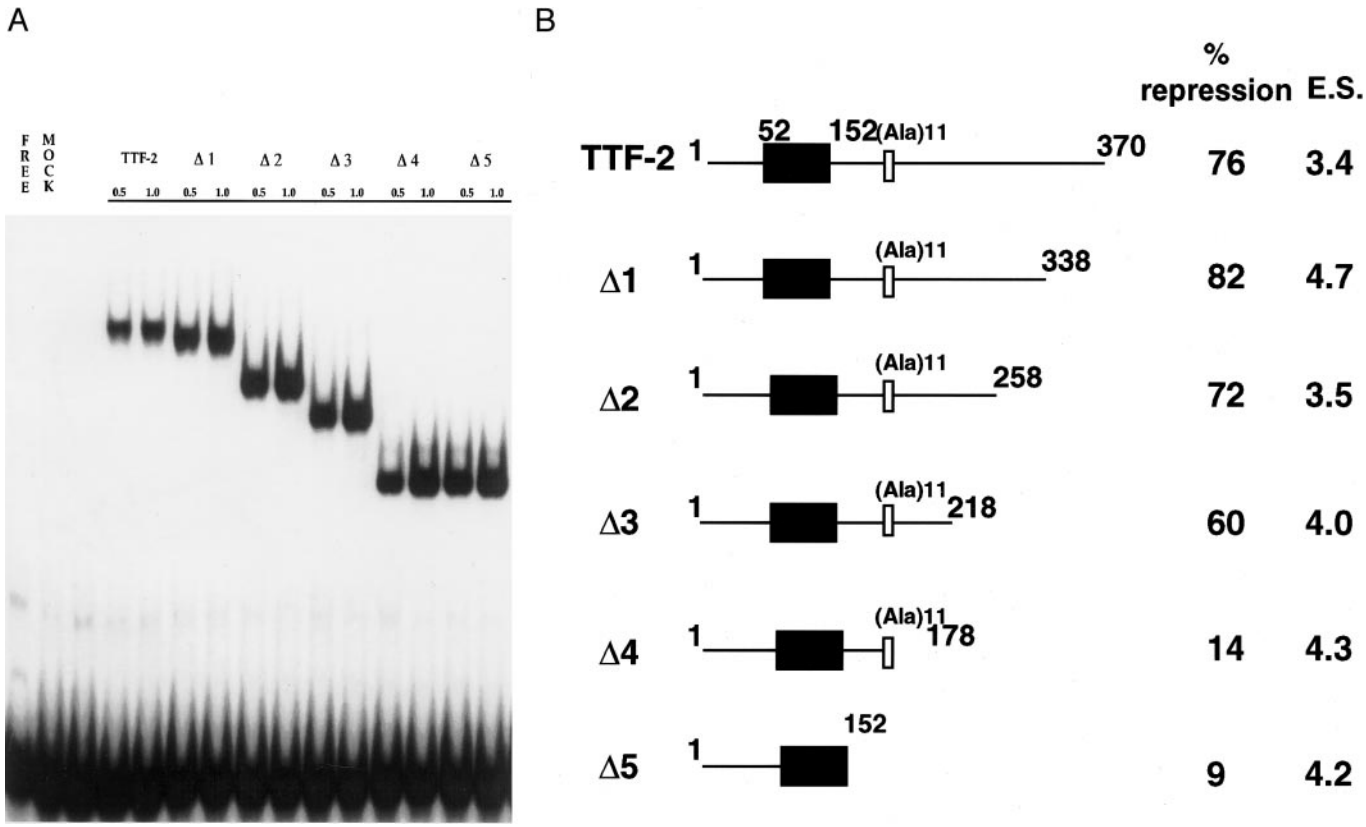

FIG. 4. Repression activity of TTF-2 deletion mutants. (A) Extracts from transfected cells were used in mobility shift assays carried out using the oligonucleotide K, which contains a high affinity TTF-2 binding site. (B) HeLa cells were transiently transfected with TgCAT (5 μg), with TTF-1 (0.1 μg), and with the deletion mutants of TTF-2 (0.5 μg). CMVluc was cotransfected to normalize for transfection efficiency. The structure of each deletion mutant is shown on the left of the figure. Numbers refers to the amino acid residue in the TTF-2 protein. On the right of the figure is indicated the repression activity of each mutant. Values are obtained subtracting the activity of the reporter in presence of TTF-2 from the activity of the reporter in absence of TTF-2 (taken as 100%). Values represent the average of at least three independent experiments.

these experiments are summarized in Fig. 4B, and led us to conclude that residue 218 represents the carboxy-terminal boundary of the repression domain. To confirm the role of this region in the repressor activity, we constructed fusion proteins containing either full length TTF-2 or a deletion mutant in frame with the DNA binding domain of LEXA, and we tested their capability to repress TTF-1 mediated activation of the Tg promoter. As shown in Fig. 5A, the DNA binding domain of LEXA alone has no effect on TTF-1 transcriptional activity, while the chimera LEXA\TTF-2 functions as a repressor. Moreover the fusion protein containing TTF-2 residue from 197 to 370 (LEXA/TTF-2Δ3) is also able to repress TTF-1. These results indicate that residue 197 represent the amino-terminal boundary of the repression domain. Thus, the region between residues 197 and 218 is responsible for the repression activity of TTF-2.

We then wanted to verify whether the LEXA:TTF-2Δ3(197–370) fusion protein maintain the promoter specificity of the entire TTF-2 protein. To this end, we tested the repression of such a chimeric protein on two reporter constructs activated by TTF-1, TgCAT and C5E1bCAT, this latter containing five DNA-binding sites for TTF-1 (13). The results of these experiments demonstrate that LEXA/TTF-2Δ3 behaves as the full-length TTF-2 protein, being able to repress TTF-1 activation on TgCAT only and not interfering with the transcriptional activation of C5E1b promoter (Fig. 5B).

DISCUSSION

In this paper, we investigated the repression activity of the thyroid-specific factor TTF-2. We demonstrate that TTF-2 is able to interfere with the transcriptional activation mediated by TTF-1 and Pax8 in a promoter specific fashion. Such a promoter specific TTF-2 mediated repression suggests that repression is not due to a direct binding between TTF-1/Pax8 and TTF-2. Interestingly, TTF-2 is able to repress the C-terminal activation domain of TTF-1, while it has no effect on the N-terminal domain, supporting the hypothesis that TTF-2 inhibits TTF-1 activity interacting with a specific cofactor and strongly suggesting that this cofactor

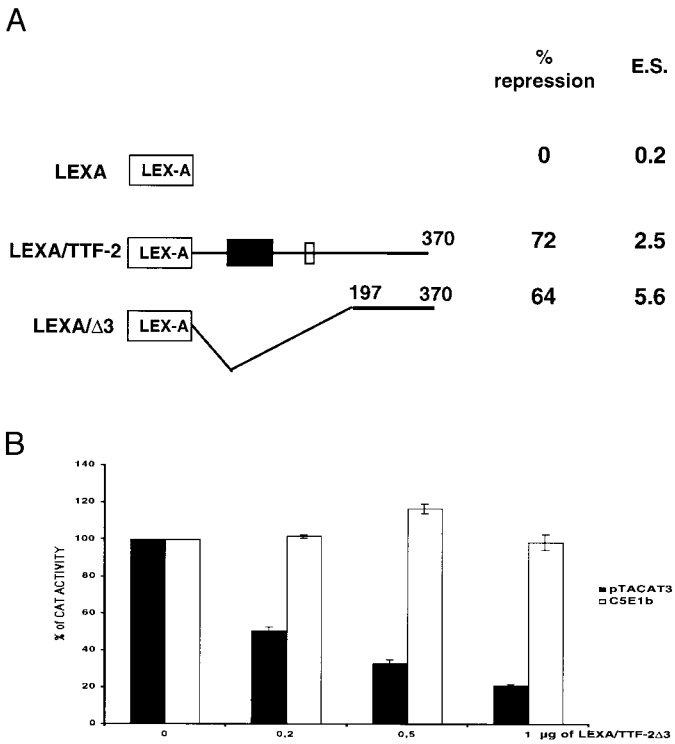

FIG. 5. TTF-2 is a DNA binding independent transcriptional repressor. (A) HeLa cells were transiently transfected with TgCAT (5 μg), with TTF-1 (0.1 μg) and with the deletion mutants of TTF-2 (0.5 μg). CMVLUC was cotransfected to normalize for transfection efficiency. On the left of the figure the structure of each chimera is shown. Numbers refer to the amino acid residue in the TTF-2 protein. On the right of the figure the repression activity of each mutant is indicated. Values are obtained subtracting the activity of the reporter in presence of TTF-2 from the activity of the reporter in absence of TTF-2 (taken as 100%). Values represent the average of at least three independent experiments. (B) HeLa cells were transiently transfected with TTF-1 (0.1 μg), with either TgCAT (5 μg) or C5E1b (5 μg) and with increasing amount of LEXA/TTF-2Δ3 (expressed as μg of DNA) as indicated. CMVLUC was cotransfected to normalize for transfection efficiency. Values are expressed as percentage of activity above that observed in absence of LEXA/TTF-2Δ3 expression (taken as 100%) and represent the average of three independent experiments. Black bars represent TgCAT activity; empty bars show C5E1b activity.

is involved in the activity of only the C-terminal activation domain of TTF-1. Moreover, we were unable to detect a direct interaction between TTF-1 and TTF-2 (data not shown); confirming that TTF-2 does not interfere with TTF-1 by a direct binding and further suggesting that the TTF-2 repressional activity involves a TTF-1-specific cofactor.

It is relevant to note that we have demonstrated that TTF-2 can repress Pax 8 activity on TPO promoter, but not on NIS promoter. These data are in agreement with previously studies, which demonstrate that Pax 8 cooperates with different molecules in the activation of the TPO and NIS promoters (14). It is tempting to speculate that cofactor(s) regulating the interplay between TTF-2 on one side and Pax8 and TTF-1 on the other are an important element in determining the switch of target genes during development. The observation that TTF-2 is able to recognize a specific promoter architecture suggests that this factor represses only a subset of the genes activated by TTF-1 and Pax 8, since all these factors are involved not only in thyroid specific gene expression (15–17), but also in thyroid morphogenesis (18–20).

We characterized the repression domain of TTF-2 using both deletion mutants in the carboxy-terminal of TTF-2 and chimeric proteins in which various portions of the TTF-2 carboxy-terminus were fused the LEXA DBD, in order to target the fusion proteins into the nucleus. The analysis of the activity of the deletion mutants of TTF-2 allowed us to map the repression domain in the region between amino acids 197 and 218 at the carboxyl terminus of the protein. As in the case of other transcriptional repressors (21–23), the repression domain of TTF-2 is rich in alanine and proline. At variance, however, of repressors such as Even-skipped and Kruppel that interact directly with TBP and the TFIIEb subunit, respectively (24, 25), we exclude for TTF-2 a repression mechanism simply based on the interference with the basal transcription machinery, since we demonstrate that TTF-2 repression is promoter specific (this paper and (1)). Interestingly, comparison of the amino acid sequence of TTF-2 repression domain with the sequences present in the database demonstrates that this region shows 41% of identity with HNF3γ amino terminal. HNF3γ is another member of the forkhead family, that plays an important role in the tissue-specific gene expression program both in early developmental and in the adult (10). Moreover, it has been demonstrated that *Genesis*, an embryonic stem cells specific forkhead containing protein, is a transcriptional repressor (26, 27). Thus, there are several lines of evidence suggesting that the forkhead containing proteins play important roles in lineage commitment and that they regulate a correct stage progression. In agreement with this hypothesis, it has been shown that TTF-2 is necessary for thyroid development, since the absence of TTF-2 expression in Titf2−\− mice impairs thyroid morphogenesis (5). In humans, mutation of the gene encoding for TTF-2 results in thyroid agenesis (28). In conclusion, the present study strongly consolidates the promoter specificity and the DNA binding independence of TTF-2-mediated repression.

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