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Transcription of the Thyroid Transcription Factor-1 (TTF-1) Gene from a Newly Defined Start Site: Positive Regulation by TTF-1 in the Thyroid

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Regulation of the thyroid transcription factor-1 (TTF-1) gene expression in the thyroid was investigated. We identified a new transcription start site as nucleotide (nt) −1917, 1700 bp upstream of previously described site, and the region encompassing nt −1242 to −14 as the first intron. Although a probe targeting exon 2 hybridized to both 3.7 and 2.7 kp transcripts, a probe targeting newly identified exon 1 mainly reacted with 3.7 kb transcript, indicating that there exsits a transcript from −1917. Chloramphenicol acetyltransferase (CAT) reporter gene assays demonstrated that 5′-flanking region of the start site exhibited promoter activity in FRTL-5 cells but not in rat liver cells, suggesting that this region confers the thyrocyte-selective expression of the gene. Two consensus TTF-1 binding motifs were detected in this promoter region, and electrophoretic mobility-shift assays showed that oligonucleotide probes, each containing one of these motifs, formed a complex with the recombinant TTF-1 homeodomain. Moreover, recombinant TTF-1 increased the transcriptional activity in FRT cells which do not express TTF-1. These results suggest that transcription from the newly identified start site in the TTF-1 gene is positively regulated by TTF-1 in the thyroid. © 1997 Academic Press

TTF-1, which belongs to a family of homeobox-containing genes of Drosophila NK2 protein (1, 2) is expressed in adult rat thyroid, lung and restricted regions of the forebrain (3). Expression of the TTF-1 gene in the thyroid of rat embryyo is first apparent 10.5 days after conception, coincident with the onset of organogenesis (3). In thyrocytes, it has been revealed that TTF-1 regulates all three genes of thyroid specific proteins, thyroglobulin, thyroid peroxidase, and thyrotropin receptor (4). So, TTF-1 palys an important role in organogenesis of the thyroid and also in the gene expression of the thyroid-specific proreins (4).

We have previously cloned rat TTF-1 gene and shown that nucleotides (nt) 332 to 1214 relative to the translation start site constitute an intron (5). The gene has also been cloned from mouse (6) and human (7) cells, and Guazzi et al. (8) and Ikeda et al. (7) reported that nt −197 is the transcription start site of the TTF-1 gene in both rat thyroid and human lung. The former group further showed that the homeobox protein HOXB3 exhibits transactivating activity at the TTF-1 gene. However, one of the TTF-1 cDNAs cloned by Mizuno et al. (9) contains a 98 bp unidentified insertion in the 5′-untranslated region, suggesting that there exists a distinct mechanism of TTF-1 gene transcription in the thyroid.

To investigate the structure and function of the TTF-1 gene, we attempted to isolate TTF-1 cDNAs from rat FRTL-5 thyroid epithelial cells. We obtained a TTF-1 cDNA whose 5′-end corresponds to nt −1902 relative to the ATG start codon, 1700 bp upstream of any previously described transcription start site. So, we determined the transcription start site of this newly identified TTF-1 transcript and studied the regulatory mechanism of TTF-1 gene expression in the thyroid.

MATERIALS AND METHODS

Determination of the transcription start sites. Rapid amplification of cDNA ends (RACE) was performed with a 5′-AmpliFINDER RACE kit (Clontech) to clone the 5′-end of the TTF-1 cDNA. Poly(A)$^+$ RNA (2 μg) from FRTL-5 cells was used to synthesize the first-strand cDNA with a TTF-1-specific primer (5′-TGTCTGTAAGCTGCGAGCGGA). After the anchor ligation, PCR was performed with a primer complementary to the anchor and a TTF-1 specific second primer (5′-TTCTTGAAGCTTTCCTCCAGGGGACTCAG) for 30 cycles of 94° for 1 min, 55° for 2 min, and 74° for 2 min. The products were subcloned into pCR II and screened with ^{32}P-labeled TTF-1 genomic sequence (nt −2200 to −2) as a probe (Nucleotide sequence of TTF-1 gene (from nt −3511 to +2148; A in the initiating ATG is designated as +1) has been entered into the DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D38035).

Primer extension analysis was performed as previously described (10) with the oligonucleotide primer 5′-CCTTAGCTGCTGATCCTGA-3′ (the antisense sequence of nt −1862 to −1842 of the TTF-1 gene) using poly(A)$^+$ RNA from FRTL-5 (ATCC CRL 8305) or BRL-3A (ATCC CRL 14429) cells.

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Plasmid construction and CAT reporter gene assays. The rat TTF-1 gene promoter from nt −2507 to −1892 was generated by PCR with Ex Taq DNA polymerase (Takara Shuzo), synthetic oligonucleotide primers, and the cloned rat gene as a template. The upstream primer included an Sph I restriction site and the downstream primer included Xba I site to facilitate cloning. The PCR product was sequenced, digested and ligated into Sph I and Xba I site of pCAT Basic reporter plasmid (Promega, Madison, WI). The resulting construct was termed pCAT-TTF-1 (−2507), and a series of 5′-deletion mutants was derived by removing various lengths of the 5′-region. pRc/CMV-TTF-1 was kindly donated Prof. R. Di Lauro, Naples, Italy.

Transient transfection was performed by electroporation (Gene Pulser, Bio-Rad) with 30 μg of pCAT-TTF-1 (−2507) (or equivalent molar mounts of the deletion mutants or pCAT Basic) together with 5 μg of pCH110 β-Gal, in order to correct for variability in transfection efficiency. Cells were pulsed at 300 V (FRTL-5 and FRT cells which were donated from Dr. L. D. Kohn, NIH) or 270 V (BRL-3A cells) at a capacitance of 960 μF, plated (6×10^6 cells per 90 mm dish), and cultured for 72 h (FRTL-5 and FRT cells) or 48 h (BRL-3A cells). All transfections were performed in triplicate with at least two different DNA preparations for each expression construct. Chloramphenicol acetyltransferase (CAT) activity was measured by the methods previously described (11, 12) using [^{14}C]chloramphenicol and its acetylated derivatives, and b-Galactosidase activity was assayed according to Sambrook et al. (13).

Electrophoretic mobility-shift assay (EMSA). Oligonucleotides used for EMSAs were as follows: oligo D (5′-AGAGAGCACTCTTCA-AGGAGAGATCTTAAA-3′, corresponding to nt −2257 to −2228 of the rat TTF-1 gene, which contains the distal consensus TTF-1 binding sequence; oligo P (5′-CAAAAACCAAAATCAAGAAGCCTCCTT-GAC-3′, corresponding to nt −2090 to −2061 of the rat TTF-1 gene which contains the proximal consensus TTF-1 binding sequence; and oligo C (5′-CACTGCCCAGTCAAGTGTTCTTGA-3′), which contains the sequence of the proximal TTF-1 binding site of the rat thyroglobulin gene promoter (14). Production of recombinant TTF-1 homeodomain protein was carried out as follows; The cDNA (222 bp), which encods the homeodomain (HD) of TTF-1 (1), was amplified by PCR with the primers, 5′-TATCTGCAGCACGCCGGAAGCGTCGGG-3′ and 5′-AGACAAGCTTCTGCTGCGCCGCC-3′, and then cloned into pTrc His B (Invitrogen). The encoded protein contains 68 amino acids of the TTF-1 HD, and is the same as the recombinant TTF-1 HD described by Guazzi et al. (1). The plasmid was then used to transform E. coli (BL21 pLysS).

EMSAs were performed as described by Shimura et al. (15). In brief, 50 ng of recombinant TTF-1 HD were incubated for 20 min at room temperature, in the absence or presence of unlabeled competitor oligonucleotide, in a final volume of 30 μl containing 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 12.5% (v/v) glycerol, 0.1% Triton X-100, and 1 μg of poly(dI-dC). End-labeled probe (50,000 cpm, 0.5 ng of DNA) was added and the mixture was incubated for an additional 20 min at room temperature. DNA-protein complexes were separated on a 6% native polyacrylamide gel.

RESULTS

Cloning and Sequencing of TTF-1 cDNA Containing the 5′ Untranslated Region from FRTL-5 Cells

To determine the 5′-end of TTF-1 mRNA, we performed RACE with poly(A)$^+$ RNA isolated from FRTL-

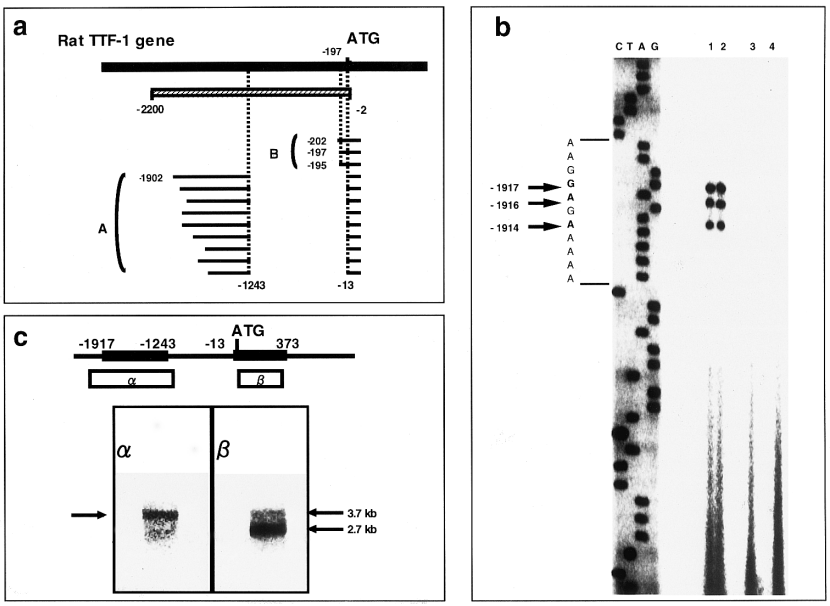

FIG. 1. (a) Schematic representation of TTF-1 cDNAs cloned by 5′ RACE. The rat TTF-1 gene is presented by a thick solid line, with the ATG translation initiation codon and the previously reported transcription start site at nt −197 (16) indicated. The genomic sequence from nt −2200 to −2, used as a probe for screening cDNA clones is shown by a hatched box. Each of the 12 cDNAs (9 type A and 3 type B) cloned by 5′-RACE is represented by a thin solid line. Nucleotides −1242 to −14 of the gene constitute an intron. (b) Determination of the 5′-end of rat TTF-1 transcript by primer extension analysis. The analysis was performed with a ^{32}P-labeled primer corresponding to nt −1869 to −1848 of the rat TTF-1 gene and either total RNA (lane 1) or poly(A)$^+$ RNA (lane 2) from FRTL-5 cells, poly(A)$^+$ RNA from BRL-3A cells (lane 3), or no RNA (lane 4). Sequencing reactions (lanes C, T, A, and G) were performed with the same primer. Transcription start sites were identified at G (−1917), A (−1916), and A (−1914) as indicated by the arrows. (c) Northern blot analysis of TTF-1 transcripts in FRTL-5 cells. Poly(A)$^+$ RNA (5mg) from FRTL-5 cells was subjected to Northern blot analysis with ^{32}P-labeled probes (α or β) corresponding to the indicated regions of the rat TTF-1 gene (first and second exon, respectively). Numbers on the schematic representation of the gene correspond to the 5′ and 3′-ends of exons. Transcripts of 3.7 and 2.7 kb are indicated (arrows).

5 cells. The amplified cDNAs were subcloned into the pCR II vector and screened with a ^{32}P-labeled TTF-1 genomic probe (nt −2200 to −2). We analyzed the 5′-ends and intron-exon organization of 12 positive clones, and a schematic representation of the cDNAs compared with the rat TTF-1 gene is shown in Fig. 1a. On the basis of intron-exon organization, we were able to classify clones into two types of transcripts, types A and B. The 5′-most nucleotide of the type A mRNAs (nine clones) corresponded to nt −1902 of the gene, 1700 bp upstream from the transcription start sites previously described (7, 8). We determined that nt −1242 to −14 of the gene, whose 5′ and 3′-ends are GT and AG, respectively, constitute an intron. The 5′-ends of type B mRNAs (three clones) corresponded to nt −202, −197, and −195 of the gene, and no introns were detected in the 5′-untranslated region of the gene with these transcripts. Thus, the type B transcripts likely correspond to that described by Guazzi et al. (8), but our results indicated that at least one of the transcription start sites of the TTF-1 gene is located much further upstream than that previously identified.

Analysis of the Transcription Start Sites of the Newly Identified TTF-1 Transcripts

To validate the results of RACE, we performed primer extension analysis. With a labeled primer corresponding to nt −1869 to −1848 of the rat TTF-1 gene, extension products were observed with total and poly(A)$^+$ RNA from FRTL-5 cells, but not with poly(A)$^+$ RNA from BRL-3A cells. The transcription start sites of this type of the TTF-1 transcripts from FRTL-5 cells were mapped to nt −1917, −1916, and −1914 (Fig. 1b). RNase protection assays, using a cRNA probe corresponding to nt −2380 to −1784 of the TTF-1 gene yielded an 190-bp broad protected band with total RNA from FRTL-5 cells but not with that from BRL-3A cells, supporting the results that the transcription start site of type A TTF-1 mRNA is located around nt −1950 of the gene (data not shown).

In order to confirm the existence of type A TTF-1 transcripts in FRTL-5 cells, we performed Northern blot analysis. Probe β comprising nt 1 to 331 of the rat TTF-1 gene hybridized to mRNAs of 2.7 and 3.7 kb, similar to previous observations (1, 9). In contrast, Probe a comprising nt −2200 to −1022, designed to be specific for type A transcripts, mainly reacted with the 3.7 kb mRNA (Fig. 1c); this transcript may correspond to the "longer transcript" previously described by Guazzi et al. (1).

Transcriptional Activity of the Promoter Region of the Rat TTF-1 Gene from the Newly Defined Start Site

To characterize the promoter activity of type A transcription of the rat TTF-1 gene, we transfected FRTL-5 or BRL-3A cells with CAT reporter gene constructs containing nt −2507 to −1892 of the TTF-1 gene or various 5′-deletion mutants thereof. In FRTL-5 cells, the promoter activities of pCAT-TTF-1 (−2507) and pCAT-TTF-1 (−2262), but not pCAT-TTF-1 (−2021) or pCAT-TTF-1 (−1990), were significantly higher than that of the control vector (Fig. 2). None of the TTF-1 gene promoter constructs exhibited transcriptional activity in BRL-3A cells. Thus, the sequence from nt −2507 to −2021 of the rat TTF-1 gene exhibits tissue-selective promoter activity.

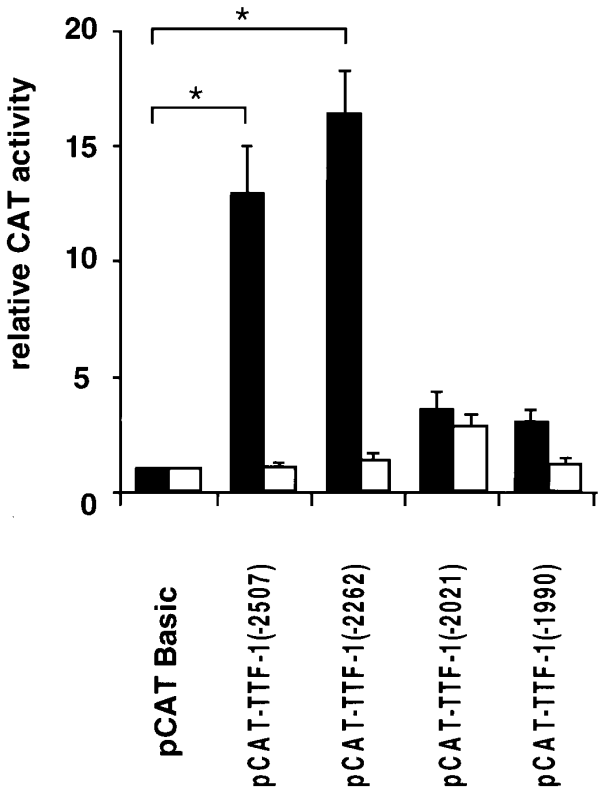

FIG. 2. Promoter activitiy of the rat TTF-1 gene. FRTL-5 (solid bars) and BRL-3A (open bars) cells were transfected with a construct containing nt −2507 to −1892 of the rat TTF-1 gene [pCAT-TTF-1 (−2507)], or various 5′-deletion mutants thereof, upstream of the CAT reporter gene. Cells were also transfected with pCH110 β-Gal to allow correction of CAT activities for variations in transfection efficiency. CAT activity is expressed relative to that of the control, pCAT Basic plasmid. Data are means ± SE of at least six experiments. *P <0.01 vs. corresponding pCAT Basic value (Student's t test).

Interactions of TTF-1 With the Newly Identified Promoter Region of the Rat TTF-1 Gene and Its Effect on Promoter Activity

Two putative TTF-1 core binding motifs (TCAAG) (16) were detected at nt −2245 to −2241 and nt −2078 to −2074 in the newly identified promoter region of the rat TTF-1 gene. We therefore investigated whether

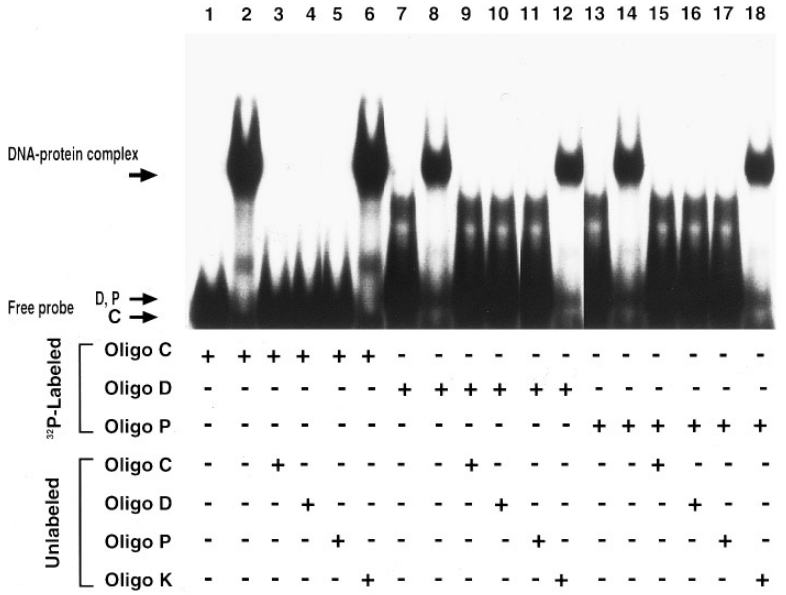

FIG. 3. Interaction of the recombinant HD of TTF-1 with oligonucleotides containing TTF-1 binding motifs from the rat TTF-1 gene promoter. ^{32}P-Labeled oligo C (containing the proximal TTF-1 binding site of the rat thyroglobulin gene promoter) or oligo D or P (containing the distal and proximal TTF-1 binding motifs of the rat TTF-1 gene promoter, respectively) were incubated with the recombinant TTF-1 HD in the absence or presence of a molar excess of unlabeled oligonucleotide as indicated. The binding mixtures were then subjected to EMSA. Lane 1, 7, and 13 correspond to reaction mixtures without the HD. A 500-fold molar excess of oligo K (5′-TGA CTAGCAGAGAAAACA-AAGTGA-3′), which contains the TTF-2 binding site of the rat thyroglobulin gene promoter (4) was used as an unrelated competitor and had no effect on DNA-protein complex formation (lins 6, 12 and 18).

TTF-1 binds to these sites by performing EMSAs with ^{32}P-labeled oligonucleotides and the recombinant HD of TTF-1 (Fig. 3). The TTF-1 HD formed a complex with ^{32}P-labeled oligo C, which contains the proximal TTF-1 binding site of the rat thyroglobulin gene promoter, and complex formation was sensitive to competition with unlabeled oligo C (Fig. 3, lanes 1 to 3). Thus, the recombinant protein had the ability to bind to the thyroglobulin gene promoter, as previously described by Guazzi et al. (1). The TTF-1 HD protein also formed a complex with ^{32}P-labeled oligo D (Fig. 3, lanes 7 and 8) and oligo P (Fig. 3, lanes 13 and 14), which contain the distal and proximal TTF-1 binding motifs, respectively, of the rat TTF-1 gene promoter; the formation of each complex was inhibited by unlabeled oligo C, oligo D, and oligo P (Fig. 3, lanes 9 to 11 and 15 to 17). The formation of the oligo C-TTF-1 HD complex was also inhibited by a 500-fold molar excess of unlabeled oligo D and 250-fold molar excess of unlabeled oligo P (Fig. 3, lanes 4 and 5).

To investigate the effect of TTF-1 on transcription of the TTF-1 gene, we co-transfected FRT cells, which contain a trace amount of Pax-8 but apparently no TTF-1 (17, 18), with pRc/CMV-TTF-1 and pCAT-TTF-1 (−2262) (which contains nt −2262 to −1892 of the rat TTF-1 gene). The pRc/CMV-TTF-1 vector increased the transcriptional activity of pCAT-TTF-1 (−2262) in a dose-dependent manner, with a maximal, three fold effect compared with the same amount of pRc/CMV (Fig. 4).

DISCUSSION

Guazzi et al. (8) and Ikeda et al. (7) previously analyzed the transcription start sites of the TTF-1 gene in

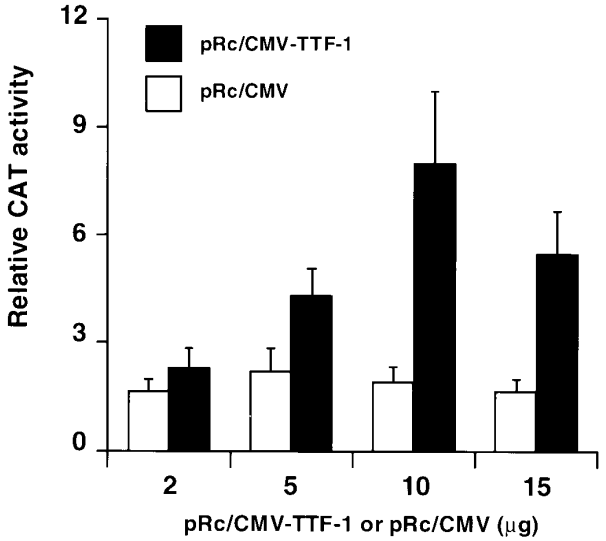

FIG. 4. Effect of TTF-1 on the transcriptional activity of the rat TTF-1 gene promoter. FRT cells were cotransfected with 30 μg of pCAT-TTF-1 (−2262) and various amounts of pRc/CMV-TTF-1 or pRc/CMV as indicated. Cells were also transfected with pCH110 β-Gal to allow normalization of CAT activities. CAT activity is expressed relative to that obtained with pCAT Basic. Data are means ±SE for three separate experiments.

rat thyroid and human lung, respectively. Both groups identified nt −197 as the site of transcription initiation. We have now identified a new type (type A) of TTF-1 transcripts in thyroid epithelial cells that differ from those previously described in that they contain a 5′ untranslated exon and are transcribed from nt −1917 of the gene.

Northern blot analysis with Probe β (nt 1 to 331) showed that FRTL-5 cells contain TTF-1 mRNAs of 3.7 and 2.7 kb, consistent with the results of previous studies (1, 7). We also showed that Probe α mainly detected the 3.7-kb transcript. Our results thus suggest that the 3.7-kb transcript might be transcribed from nt −1917.

Mizuno et al. (9) previously identified TTF-1 transcripts from the rat thyroid that contain a 98-bp insertion in the 5′-untranslated region. On the basis of RNase mapping data, Guazzi et al. (1) also suggested that alternative transcription initiation sites, upstream of nt −197, might exist in the lung. However, the sequence data presented in both of these previously studies were not sufficient to assess the relation of the corresponding mRNAs with type A transcripts described here.

Nucleotides −339 to −95 (previously referred to as nt −142 to +103) have been shown to regulate transcription from nt −197 of the rat TTF-1 gene (8). HOXB3 binds to two ATTA sites located in this promoter region and exhibits trans-activating activity (8). We have now identified nt −2507 to −2021 as important for transcription from nt −1917. CAT reporter gene assays also revealed that this region of the TTF-1 gene is important for thyroid-selective expression. This promoter region contains two consensus TTF-1 binding motifs (TCAAG) at nt −2245 to −2241 and nt −2078 to −2074. EMSAs revealed that these two sites bind the recombinant TTF-1 HD. Moreover, TTF-1 increased the transcriptional activity of the promoter region of the TTF-1 gene containing these two binding motifs. Homeotic genes of Drosophila (19) and the mammalian homeodomain-containing Pit-1 gene (20, 21) are also autoregulated by their respective protein products, supporting our results.

It will be needed to clarify the physiological role the TTF-1 transcript from −1917 in gene expression of thyroid-specific proteins, but our results indicate that the mechanism of transcriptional regulation for transcripts initiated at nt −1917 differs from that for those initiated at nt −197. Further analysis of these mechanisms should contribute to our understanding of the role of TTF-1 in the pathophysiology of the thyroid gland.

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