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The Interaction between the Forkhead Thyroid Transcription Factor TTF-2 and the Constitutive Factor CTF/NF-1 Is Required for Efficient Hormonal Regulation of the Thyroperoxidase Gene Transcription*

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The forkhead thyroid-specific transcription factor TTF-2 is the main mediator of thyrotropin and insulin regulation of thyroperoxidase (TPO) gene expression. This function depends on multimerization and specific orientation of its DNA-binding site, suggesting that TTF-2 is part of a complex interaction network within the TPO promoter. This was confirmed by transfection experiments and by protein-DNA interaction studies, which demonstrated that CTF/NF1 proteins bind 10 base pairs upstream of the TTF-2-binding site to enhance its action in hormone-induced expression of the TPO gene. GST pull-down assays showed that TTF-2 physically interacts with CTF/NF1 proteins. In addition, we demonstrate that increasing the distance between both transcription factors binding sites by base pair insertion results in loss of promoter activity and in a drastic decrease on the ability of the promoter to respond to the hormones. CTF/NF1 is a family of transcription factors that contributes to constitutive and cell-type specific gene expression. Originally identified as factors implicated in the replication of adenovirus, this group of proteins (CTF/NF1-A, -B, -C, and -X) is now known to be involved in the regulation of several genes. In contrast to other reports regarding the involvement of these proteins in inducible gene expression, we show here that members of this family of transcription factors are regulated by hormones. With the use of specific CTF/NF1 DNA probes and antibodies we demonstrate that CTF/NF1-C is a thyrotropin-, cAMP-, and insulin-inducible protein. Thus CTF/NF1 proteins do not only mediate hormone-induced gene expression cooperating with TTF-2, but are themselves hormonally regulated. All these findings are clearly of important value in understanding the mechanisms governing the transcription regulation of RNA polymerase II promoters, which often contain binding sites for multiple transcription factors.

The mechanisms by which cells selectively activate the transcription of a specific gene are essential. Tissue-specific transcription factors that bind DNA sequences within the promoter are the main mediators of tissue-specific gene expression (1). It has become clear, however, that transcriptional activation of a given gene is defined not only by the activity of an individual factor or a single DNA-binding site, but rather, depends on combinatorial interactions between multiple proteins (2, 3). To understand the mechanism of tissue-specific transcriptional activation, it is first necessary to identify *cis*-regulatory elements and to characterize tissue-specific transcription factors, and then to define protein-protein interaction that determine their function.

The focus of our work has been to understand the regulatory mechanisms underlying hormonal transcription of the thyroperoxidase (TPO)[1] gene, a tissue-specific enzyme expressed only in differentiated thyroid cells. Its function involves the iodination and coupling of tyrosine residues into the thyroglobulin molecule to generate thyroid hormones (4–6). Both thyroglobulin and TPO are cell type-specific genes whose respective promoters have been characterized (7, 8). With the use of DNA binding assay, three thyroid-specific transcription factors have been identified: TTF-1, TTF-2, and Pax-8 (9). Cloning of these three proteins demonstrated that they are members of different transcription factor families. TTF-1 and Pax-8 are homeo- and paired-containing proteins, respectively (10, 11), and TTF-2 is a forkhead protein (12). The three factors are expressed at the beginning of thyroid development (11–13) and are considered decisive in the maintenance of thyroid phenotype. All of them bind within the thyroglobulin and TPO promoter at similar positions (7, 8, 14) although the TPO promoter differs in several aspects from that of thyroglobulin. It is thus approximately an order of magnitude less active than the thyroglobulin promoter; the Pax-8 protein overlapping the TTF-1-binding site has a different position (14) and the ubiquitous transcription factors that bind to both promoters occupy different sites

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[1] The abbreviations used are: TPO, thyroperoxidase; TSH, thyrotropin; GST, glutathione *S*-transferase; CAT, chloramphenicol acetyltransferase; bp, base pair(s); RSV, Rous sarcoma virus; kb, kilobase(s); EMSA, electrophoretic mobility shift assay; MMTV, mouse mammary tumor virus; PAGE, polyacrylamide gel electrophoresis; TTF-2, thyroid-specific transcription factor 2; UFB, ubiquitious factor B.

in each one (7, 8).

Several ligands regulate the expression of the TPO gene through alteration of the activity of the transcription factors that control its expression. For example, we have recently demonstrated that the transcription factor TTF-2 is under the hormonal control of the thyrotropin (TSH) and the cAMP as well as to the insulin and insulin-like growth factor I signaling pathways (15). TTF-2 binds to a single site that acts as a hormone response element. This function depends on multimerization and specific orientation of the TTF-2-binding site (16). This suggests that TTF-2 is part of a complex interaction network within the TPO promoter, whose final result is to turn-on the specific expression of the TPO gene in response to external hormonal stimuli.

As the binding site for TTF-2 functions in an orientation-specific manner, we asked whether TTF-2 alone regulates the expression of this gene or requires the action of neighboring sequences. Neighboring regulatory elements of TTF-2 bind the thyroid-specific transcription factor TTF-1 and the ubiquitous transcription factor UFB (8). Here we show by transient transfection assays and site-directed mutagenesis that the binding sites for TTF-2 and UFB are important for hormone-induced expression at the TPO promoter. Furthermore, we show that UFB is a binding site for members of the CTF/NF1 family of transcription factors. These are a multiprotein family in which four different genes have been cloned, NF1-A, NF1-B, NF1-C, and NF1-X (17–19), as well as different isoforms generated by alternative splicing (17, 20–22). Although their expression is fully ubiquitous, differences can be detected in the distribution and abundance of their different transcripts (23, 24). Strikingly, we have found that, as occurs for TTF-2 (15), CTF/NF1 gene expression is up-regulated by TSH and insulin in inducing the expression of the TPO gene. Furthermore, we present evidence, from a GST pull-down assay, that these constitutive factors interact physically with TTF-2. This interaction appears to be functional, since the TPO promoter activity and its hormonal response are lost in transfection experiments in which the distance between the CTF/NF1 and TTF-2-binding site has been altered. Thus the hormonal control of TPO gene transcription, which takes place exclusively in thyroid-differentiated cells, depends on the correct stereospecific interaction of two hormonally expressed transcription factors: TTF-2 and CTF/NF1.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium, bovine TSH, and bovine insulin were purchased from Sigma, and forskolin from Roche Molecular Biochemicals (Mannheim, Germany). Donor, fetal calf serum, and Dulbecco's modified Eagle's medium were from Life Technologies, Inc. (Gaithersburg, MD); Nytran membranes were obtained from Schleicher & Schüll (Richmond, CA). TnT and luciferase assay kits were purchased from Promega (Madison, WI). [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{35}S]methionine were from ICN (Irvine, CA).

Cell Culture and Transfection—FRTL-5 cells (ATTC CRL 8305; American Type Culture Collection, Manassas, VA) were cultured as described previously (25) in Coon's modified Ham's F-12 medium supplemented with 5% donor calf serum and a six-hormone mixture including 1 nM TSH and 10 μg/ml insulin (complete medium). The effect of TSH and insulin were studied by starving confluent or transfected cells for both hormones in the presence of 0.2% serum (basal medium) (26). After 4 days, each ligand was added to the culture medium at the concentrations given. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For transient expression assays, transfections were performed by the calcium phosphate coprecipitation method, as described for each cell line (16, 27). The plasmid RSV-CAT (28) was used to correct for transfection efficiency. Luciferase and CAT activities of cell extracts were determined as described (29, 30).

Promoter Constructs—p420 TPO LUC containing the minimum TPO promoter linked to the luciferase cistron (8) and the deletion p60 TPO LUC (16) have been previously described. The deletions p120 and p92 TPOLUC, as well as insertions p120(+5) and p120(+10), were generated by the polymerase chain reaction on the p420 TPO LUC template, using as flanking primers the 3′ polymerase chain reaction primer LUC-1: 5′-GGATAGAATGGCGCCGGGCCTTTCTTTATG-3′ and the following oligonucleotides as 5′ polymerase chain reaction primers: TPO-120, 5′-AAGAGCTCATACTAAACAAACAG-3′; TPO-92, 5′-AAGAGCTCGACACACAAGCACTTGGCAG-3′; TPO-120 (+5), 5′-AAGAGCTGACACACAAGCACTTGGCAGAAACGGATCAAATACTAAAC-3′; and TPO-120 (+10), 5′- AAGAGCTGACACACAAGCACTTGGCAGAAACGGATCCGACGAAATACTAAAC-3′. Amplified fragments were digested with *Sac*I and *Pst*I and subcloned into pBS LUC-2 (8). The mutated constructs, pBmm TPO LUC and pZm TPO LUC were previously described (8). In the pBmm mutant the TTGG sequence is altered to GGTC and TTF-1 and UFB binding activity is thus undetectable. pZm contains a 4-bp mutation within the Z site, which interferes with TTF-2 binding. Constructs containing tandem repeats of Z site (−93 to −73) in front of the minimal promoter TATA LUC have been described elsewhere (16). The pBZ TATA LUC was made by insertion of a double strand synthetic oligonucleotide containing the BZ (−118 to −73) region of the TPO promoter 5′ → 3′, into the *Sma*I site of the plasmid TATA LUC (16).

Expression Vectors—pSG-LexVP-16 has been previously described (31). pBAT-hCTF-1 and pBAT-CTF/NF1-X constructs were obtained by digesting the coding sequence of NF1/CTF1 and NF1-X from the RSV-based expression vector (21) with *Xba*I/*Xho*I and *Eco*RI/*Xba*I followed by a fill-in reaction and ligation into the *Sma*I site of the plasmid pBAT (32). The bacterial expression plasmid pGEX-4T3 was utilized to direct overexpression of the full-length GST-TTF-2 fusion protein. The full-length TTF-2 cDNA was digested with *Bam*HI and *Eco*RI and subcloned into the pGEX-4T3 vector. The mammalian expression vector RSV-CTF/NF1-C has been previously described (21). The expression vectors CMV-CTF/NF1-B and CMV-CTF/NF1-X (33) were kindly provided by Dr. B. Gao (MCV-VCU, Richmond, VA).

RNA Analysis—Total RNA was extracted by the guanidinium isothiocyanate procedure (34). Polyadenylated RNA preparation was performed with oligo(dT)-cellulose chromatography as described by Nebl *et al.* (35). Thirty micrograms of total RNA or 5 μg of poly(A)$^+$ were denatured and fractionated on a 1% agarose gel containing 3.7% formaldehyde. RNA was then blotted and fixed onto Nytran membranes. The radioactive probes used included a 0.35-kb *Hin*dIII/*Eco*RI fragment from the 3′-untranslated region of rat TTF-2 (p3′UTRT) (12), a 0.6-kb *Eco*RI fragment from rat TTF-1 (10), a 1.1-kb *Eco*RI fragment from the 5′-end of human CTF-1 (17), a 0.5-kb *Pst*I/*Eco*RI fragment from the 5′-end of the hamster NF-1 X (18), a 1.5-kb *Eco*RI from the 5′-end of hamster NF-1/Red (18), and a 1.0-kb *Pst*I fragment of p91 α-actin (36). Hybridizations were carried out at 65 °C in 4 × SSC (1 × SSC is 0.15 M NaCl, 0.125 M sodium citrate), 10 mM EDTA, and 0.05% SDS. After hybridization, the filters were washed at 65 °C for 30 min each in 3.3% phosphate buffer, pH 7.2, 0.1% SDS and successively lower salt concentrations (2, 1, and 0.5 × SSC) before autoradiography.

DNase I Footprinting and Electrophoretic Mobility Shift Assays—For electrophoretic mobility shift assays (EMSA), the nuclear protein fraction was extracted by the procedure described by Andrews and Faller (37). Protein concentration was measured according to Bradford (38) with the Bio-Rad protein assay kit using bovine serum albumin as standard. The recombinant purified NF-1 protein, used in both EMSA and DNase footprinting, was kindly provided by Dr. M. Beato (Institut fur Molecularbiologie und Tumorforshung, Marburg, Germany). The footprinting probe, corresponding to the −257 to +30 region of the TPO promoter was prepared by polymerase chain reaction using TPOF1 (−257 to −236): 5′-ATAAGAGAAACTCCCAGGAACC-3′, and TPOF6 (+9 to +30): 5′-ACTTCAGAAATGTGAATCTCAA-3′ labeled oligonucleotides as flanking primers on the p420 template. DNase I footprinting reactions were carried out in a 50-μl reaction volume as follows: recombinant NF-1 (5 or 25 ng) was preincubated with 5 × 10^4 cpm for 45 min on ice in 20 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, and 0.4 mg/ml bovine serum albumin. DNase I digestion was performed by the addition of 6 ng of DNase I in 10 mM $MgCl_2$, 2 mM $CaCl_2$ and incubation for 1 min at room temperature. Footprinting reactions were terminated by addition of 200 μl of a stop mixture (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA, 0.5% SDS, and 250 μg/ml proteinase K). The samples were incubated for 1 h at 45 °C, extracted with phenol-chloroform, ethanol precipitated, and resuspended in formamide dye. Equal number of counts per sample were loaded and resolved on an 8% sequencing gel, together with G and G + A chemical sequencing reactions. Gels were fixed, dried, and visualized by autoradiography. EMSA were carried out as described previously (39) using ^{32}P-labeled double-stranded oligonucleotide UFB

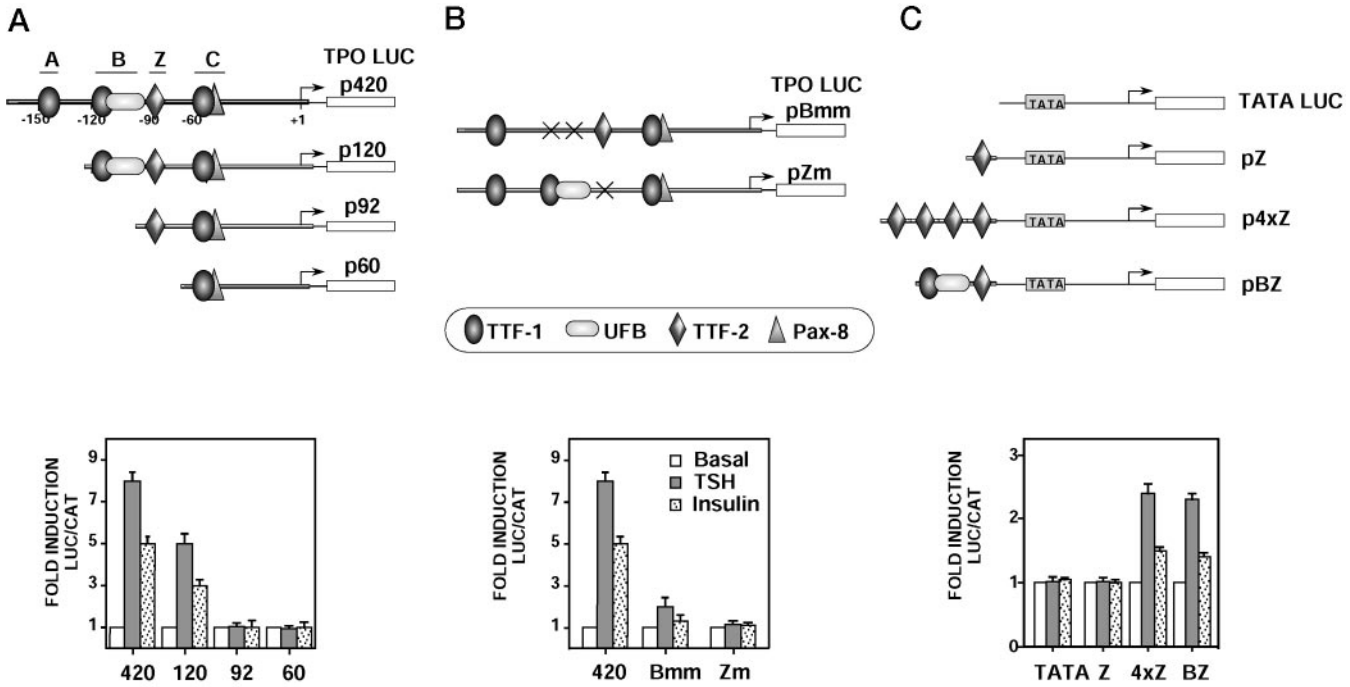

FIG. 1. **Identification of the B-site of the TPO promoter as the element that cooperates with the TTF-2 binding site.** *A-C, upper panels,* are the schematic diagram of the wild type TPO promoter (p420 TPO) and the different constructs, described under "Experimental Procedures," linked to the luciferase reporter gene (*LUC*). The deletions and mutations generated on the wild type promoter are represented as a *double line* (*panels A* and *B*) while the tandem repeats generated on the TATA LUC are represented as a *single line* (*panel C*). The protein-binding site detected by footprinting assays (8) and the corresponding transcription factors are indicated with different symbols. *A-C*, *lower panels,* correspond to the TPO promoter activity derived from 10 μg of each construct transiently transfected to FRTL-5 thyroid cells. After transfection cells were maintained 72 h in the absence of serum (0.2%) without TSH and insulin (basal medium). Then, the cells were treated with 1 nM TSH or 2 μM insulin for 24 h. Relative luciferase activity is the value of light units normalizing the results to CAT activity derived from 2 μg of RSV-CAT transfected to correct for transfection efficiency. The TPO promoter activity is expressed as fold induction over the basal levels (= 1) of hormone-depleted cells. The results are the mean ± S.D. of four independent experiments.

(5′-CAAGCACTTGGCAGAAACAAATAC-3′) or consensus NF-1 (5′-CATATTGGCTTCAATCCAAA-3′) derived from the MMTV promoter (40). For supershift experiments, 1 μl of anti-NF-1/8199/2902 (kindly provided by Dr. N. Tanese, New York University, New York) or preimmune serums were added to the preincubation mixture.

GST-TTF-2 Fusion Protein and Pull-down Experiments—An overnight culture of the bacterial strand BL21 cells harboring plasmid pGEX-4T3 or pGEX-TTF-2 was diluted 1:10 in a 2 × YT medium plus ampicillin, pH 7.0, and cultured at 27 °C to optical density at 600 nm of 0.6–0.75. Protein expression was then induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside and cultures were incubated for an additional 2 h at the same temperature. Cells were harvested and the fusion proteins purified as described (41). The integrity of the GST fusion proteins bound to the beads was analyzed by resolution by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining. Known amounts of bovine serum albumin were included on the same gel for determination of the yield. For *in vitro* translation in reticulocyte lysate, we used a coupled transcription/translation system (TnT, Promega) in the presence of [^{35}S]methionine (1000 mCi/mmol). The protocol for the GST pull-down assay was essentially as described (42). GST or GST-TTF-2 proteins (5 μg) immobilized on glutahione-Sepharose 4B beads were washed extensively with LBST-100 buffer (25 mM Hepes-KOH, pH 7.9, 100 mM NaCl, 6% glycerol, 5 mM MgCl, 1 mM dithiothreitol, 0.05% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA) and the volume was raised to 180 μl with LBST-100 buffer. Radioactively labeled protein (20 μl) was then added and gently mixed at room temperature for 30 min, followed by a further 30-min incubation with gentle shaking at 4 °C. The beads were washed four times with successively increasing NaCl concentrations (LBST-100, LBST-300, and LBST-500), and the bound proteins were analyzed in 8% SDS-PAGE followed by autoradiography.

RESULTS

Cooperativity of Regulatory Elements on the TPO Promoter—Previous results from our laboratory have demonstrated that TPO promoter activity is hormonally regulated by TSH through the cAMP pathway, as well as by insulin and insulin-like growth factor I. This regulation is mediated mainly by the *cis*-regulatory element (Z) to which the forkhead thyroid transcription factor-2 (TTF-2) binds (see Fig. 1*A*, for cartoon). Further analysis showed that the TTF-2-binding site acts as a hormone response element in a heterologous construct and that it requires a specific orientation for activation of the TPO promoter (16). This suggests that TTF-2 may require other bound factors for activation of TPO expression gene.

To identify the regulatory elements on the TPO promoter that cooperate with the TTF-2-binding site, we transfected into FRTL-5 thyroid cells different promoter constructs of this gene (Fig. 1, *upper panels*) linked to the coding region of the firefly luciferase gene (30). The transfected cells were cultured for 72 h in a minimal medium depleted of TSH and insulin but supplemented with 0.2% serum to ensure only a basal expression of the TPO (16, 43). TPO promoter activity was enhanced by treatment of the cells with TSH or insulin for 24 h. The promoter activity was determined by luciferase activity measurements while CAT activity derived from a co-transfected RSV-CAT construct was used to correct for variability in transfection efficiency.

In this transfection assay, only the p420 TPO LUC and p120 TPO LUC constructs showed hormone inducibility (Fig. 1*A, lower panel*). Neither p92 TPO-LUC nor p60 TPO-LUC, in which successive deletions were performed of the B and Z site, respectively, responded to TSH and insulin treatment. As both inducers enhanced the promoter activity of construct p120 but not p92 TPO LUC, the B element missing in the p92 construct must be important for the hormone response.

The B element has been previously reported to bind the transcription factors TTF-1 and UBF. Mutations introduced into both binding sites (pBmm) that abolished binding by their

respective factors (8) drastically reduced hormone regulation of expression at the TPO promoter (Fig. 1*B*). Mutations in the Z regulatory element destroying the binding of TTF-2 (pZm) also abrogated hormone inducibility (Fig. 1*B*) indicating a concerted action of the B and Z regulatory sites for hormone inducibility. The fact that the multimerization of the Z element confers hormone inducibility to the minimal promoter TATA LUC and a single Z element was unable (Fig. 1*C*) implies that the hormone regulatory activity of the Z element comes from cooperative action with itself or possibly with other transcription factor. To find out whether the B regulatory element can confer hormone inducibility to a single Z element, we cloned the B and Z regulatory units in front of the minimal promoter. This construct in the transfection experiments produced an identical result as p4xZ in its response to hormone treatment. We therefore concluded that the hormonal response of the TPO promoter activity is dependent on an active cooperation between TTF-2 and the factors binding to the B site. It is important to mention that in the above study the TSH effect was mimicked by forskolin (data not shown).

CTF/NF1 Proteins Bind to UFB-binding Site—Analyses of the BZ region (−120 to −73) of the TPO promoter by *in vitro* footprinting with nuclear extracts from FRTL-5 thyroid and non-thyroid Rat-1 cells had identified three different transcription factors that bind to this sequence (8). Two of these are thyroid-specific and were identified as TTF-1 and TTF-2, homeo and forkhead domains containing proteins, while the third factor was characterized as a ubiquitous factor (UFB) because it was found in both cell lines studied. Since UFB has not yet been characterized, we analyzed the B promoter region for potential recognition sequences for known ubiquitous transcription factors. At position −100 bp from the transcription initiation site within the B region is a TTGGCA motif that has been characterized as a CTF/NF1-binding site in many eukaryotic promoters (18, 44–49). Recombinant CTF/NF1 protein was therefore used in DNase I footprinting experiments with the labeled TPO promoter fragment. The purified CTF/NF1 protein protected the sequence 5′-AAGCACTTGGCAGAAACAA-3′ from position −112 to −95 (Fig. 2*A*), corresponding exactly to the previously defined UFB-binding site (8). In EMSA in which we used the UFB oligonucleotide (−112 to −95) and the recombinant CTF/NF1, two protein complexes were formed possibly due to the intrinsic property of the CTF/NF1 proteins to form homo- and heterodimers (20, 50) (Fig. 2*B, lane 2*). Both protein-DNA complexes were specifically inhibited by addition of a 100-fold excess of unlabeled oligonucleotide UFB (*lane 3*) as well as by a CTF/NF1 consensus sequence, derived from the mouse mammary tumor virus (MMTV) promoter (51) (*lane 4*) and by the BZ oligonucleotide (−120 to −73 bp of the TPO promoter) (*lane 5*), but not by an unrelated oligonucleotide (*lane 6*). If instead of the recombinant CTF/NF1 protein, nuclear extracts of FRTL-5 cells were used, a smear containing two distinct complexes was obtained (Fig. 2*C, lane 2*). These two complexes as well as the smear were also competed by a 100-fold excess of unlabeled UFB (*lane 3*) and the consensus CTF/NF1 oligonucleotide (*lane 4*) but not by an unrelated oligonucleotide (*lane 5*). Preincubation of the binding reaction with an anti-CTF/NF1 antiserum but not with a preimmune serum, produced a supershifted band with a corresponding reduction in the CTF/NF1 complexes (Fig. 2*C*, compare *lanes 6* and *7*). As a control of the supershift assay the labeled oligonucleotide was preincubated with the CTF/NF1 antibody alone (*lane 8*) but this failed to produce the supershifted band. A high molecular weight complex that barely entered the gel was formed between the antibody and the labeled oligonucleotide (Fig. 2*C, lane 8*). This comes from an interaction of the antibody with the labeled DNA. Together, these data give sufficient evidence to conclude that the UFB binding factor belongs to the CTF/NF1 family of transcription factors.

CTF/NF1 Family Members Enhance TPO Promoter Activity—Mutation of the TGGCA motif, identified in the present work as a CTF/NF1-binding site, is reported to decrease TPO promoter activity (8). We have also shown that deletion or mutations in the B regulatory element down-regulate hormone inducible expression of TPO (Fig. 1, *A* and *B*). To determine the functional activity of CTF/NF1 factors binding to the B regulatory element of the TPO gene, transient transfection experiments were carried out in HeLa cells. In these experiments, promoter constructs of the TPO gene as well as the mutant version pBmm (Fig. 3, *upper panel*) were co-transfected with expression vectors coding for various members of CTF/NF1 family such as CTF/NF1-C, -B, or -X. A 5-fold increase in TPO promoter activity was obtained in cells co-transfected with CTF/NF1-C or -X whereas CTF/NF1-B showed no effect (Fig. 3, *lower panel*). In the transfection, combinations of CTF/NF1-C and -X but not -C and -B or -B and -X additively increased the TPO promoter activity. This differential transactivation may be contributed by the ability of the CTF/NF1 proteins to bind DNA and function as homodimers and heterodimers (19, 20, 52). The pBmm TPO LUC construct, which contains a mutated TGGCA motif (8), did not show an increased transactivation after co-transfection of the different CTF/NF1 isoforms (Fig. 3). These analyses demonstrate that binding of CTF/NF1 proteins to the B regulatory region can functionally activate this promoter.

CTF/NF1 Isoforms Are Hormonally Regulated in FRTL-5 Cells—The contribution of the B regulatory element in the TPO promoter to induce expression in the presence of TSH and insulin presupposes that either one of the two or both factors binding to this region could be hormonally regulated. We have already shown that TTF-2 expression is controlled by TSH and insulin (15). To investigate whether CTF/NF1 is also regulated by these hormones, we performed Northern blot studies with poly(A)$^+$ RNA from FRTL-5 cells maintained for 4 days in a minimal medium and then treated for 24 h with 1 nM TSH, 2 μM insulin, or both hormones together.

As we have previously shown CTF/NF1-B and -X are expressed as 8.6- and 5.1-kb transcripts, respectively, whereas CTF/NF1-C is expressed as two transcripts of 6.5 and 4.0 kb (35). The relationship between the 6.5- and 4.0-kb transcripts is not too clear but it is thought that the 6.5-kb is a primary transcript which is later processed to the 4.0-kb transcript (35). TSH and insulin showed an interesting regulation of expression of the CTF/NF1-C gene. These hormones slightly reduced the level of the 6.5-kb transcript but drastically increased the level of the 4.0-kb transcript (Fig. 4, *lanes 1–3*). With the other CTF/NF1 transcripts, TSH slightly enhanced and insulin down-regulated the level of expression of the CTF/NF1-B gene while no significant effect on the level of NF1-X was observed. The same Northern blots were examined for the expression of TTF-1 and -2 as well as the control α-actin gene (Fig. 4). The expression of TTF-2 as in the case of the 4.0-kb transcript of CTF/NF1-C was strongly enhanced by both TSH and insulin (Fig. 4, *lanes 5–7*). A comparison of the expression of the CTF/NF1 and TTF-2 genes in the non-induced state and the fully induced state show that the CTF/NF1-C and TTF-2 may contribute significantly to the hormonal regulation of the TPO expression (Fig. 4, compare *lane 1* with *4* and *5* with *8*). The TTF-1 and α-actin mRNA levels were the same in the different experimental approaches studied.

As CTF/NF1 genes are known to be constitutively expressed, the observation that they may be hormonally regulated de-

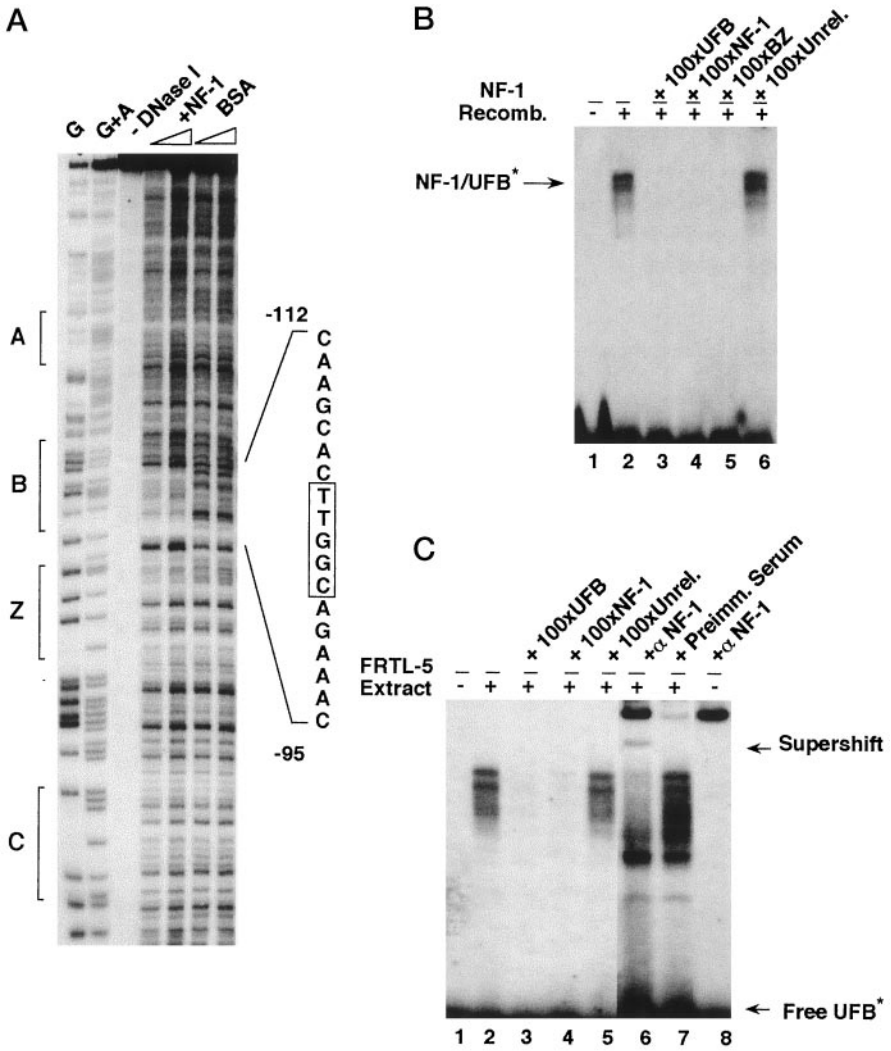

FIG. 2. **Identification of UFB nuclear factor as a member of the CTF/NF1 family of constitutive transcription factors.** *A*, DNase I footprinting analysis was performed on the −257 to +30 TPO promoter fragment with 5 (*lane 4*) or 25 (*lane 5*) ng of recombinant CTF/NF1 protein, or with an increased amount of bovine serum albumin (*lanes 6* and *7*). *Lane 1* shows the DNase I digestion of the probe in the absence of protein, and *lane 2* is the G + A reaction. The sequence of the protected region (−112 to −95) is represented, and the CTF/NF1 motif is marked with a *box*. The *A, B, Z*, and *C brackets* correspond to the protected regions previously identified with nuclear extracts of FRTL-5 thyroid cells (8). *B,* EMSA of 5 ng of CTF/NF1 recombinant protein (*lane 2*) and the protected UFB sequence identified and represented in *panel A*. For competition, a 100-fold excess of the same unlabeled oligonucleotide (*lane 3*), the consensus CTF/NF1 sequence of the MMTV promoter (*lane 4*), the BZ oligonucleotide derived from the TPO promoter (*lane 5*), or an unrelated oligonucleotide (*lane 6*) were used. *C,* electrophoretic mobility shift assay with 5 μg of nuclear extract from FRTL-5 cells and the UFB oligonucleotide (*lane 2*). Competition was done with 100-fold excess of the unlabeled oligonucleotide UFB, with the CTF/NF1 consensus sequence or with an unrelated oligonucleotide (*lanes 3–5*, respectively). The supershift assay was performed with the specific antibody (1/8199) α-NF1 (*lane 6*). The preimmune serum (*lane 7*) and the antibody alone without nuclear extracts (*lane 8*) were used as control.

serves further consideration. We therefore asked whether our findings at the mRNA level reflect changes at the level of the CTF/NF1 proteins bound to the UFB sequence of the TPO promoter. EMSA was performed with an oligonucleotide derived from the UFB site of the TPO promoter and nuclear extracts from non-treated and hormone-treated FRTL-5 cells. With extracts derived from FRTL-5 cells in the absence of hormone, two prominent protein-DNA complexes were formed which are indicated as 2 and 3 (Fig. 5*A, lane 2*). Faster migrating complexes were also observed but these were not consistently seen and may possibly have arisen as a result of minor degradation of the CTF/NF1 protein. The DNA-binding domain of the CTF/NF1 is known to be easily cleaved from the rest of the protein (46). EMSA carried out with extracts of cells treated with TSH and insulin showed quantitative and qualitative differences. In the presence of TSH there was a shift in mobility of complexes 2 and 3 to complexes 1 and 2 as well as an increase in the intensity of complex 2 (Fig. 5*A, lane 3*). Insulin treatment enhanced the intensity of complex 3 without showing any qualitative changes in the complexes compared with the pattern with extracts from non-stimulated cells (Fig. 5*A, lane 4*). A combined treatment with TSH and insulin produced complexes qualitatively and quantitatively indistinguishable from treatment with TSH alone (Fig. 5*A* compare *lane 5* with *lane 3*). Thus TSH treatment appears to play a dominant role in the changes that take place in the composition of the CTF/NF1 in the regulation of the TPO promoter activity. The complexes were specifically competed by addition of a 100-fold excess of unlabeled UFB oligonucleotide (*lane 7*) but not by an unrelated one (*lane 6*).

To confirm the ability of TSH to regulate expression of CTF/NF1 proteins, we used the same extracts from the FRTL-5 cells in an EMSA with a consensus CTF/NF1 oligonucleotide derived from the MMTV (Fig. 5*B, lanes 1–6*). These results were identical to those obtained with the UFB oligonucleotide as TSH again caused a retardation of the complexes whereas insulin

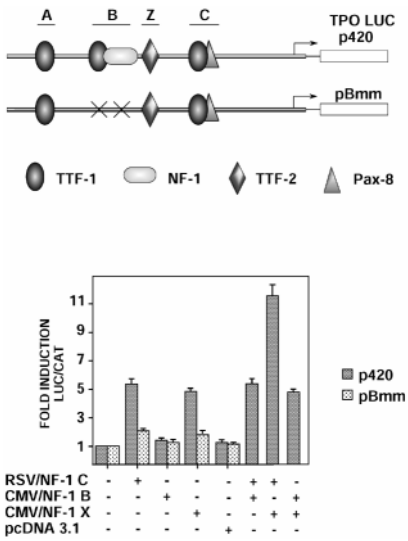

FIG. 3. **Role of the CTF/NF1 transcription factors in TPO promoter activity.** Constructs containing the wild type TPO promoter (p420 TPO LUC) and the mutation generated at the B site (*pBmm*) (*upper panel*) were co-transfected into HeLa cells with the expression vector pcDNA3.1 with no insert or harboring the cDNA for CTF/NF1-C, CTF/NF1-B, or CTF/NF1-X, as described under "Experimental Procedures." Luciferase activity (*lower panel*) was determined as relative light units normalizing to CAT activity derived from the RSV-CAT transfected to adjustments of transfection efficiency. The TPO promoter activity is expressed as fold induction over the wild type promoter (= 1). The results are the mean ± S.D. of four independent experiments.

did not have much of an effect. These results together confirm that the composition of the CTF/NF1 proteins was altered by TSH. In the Northern blot as well as in the EMSA the TSH effect was mimicked by forskolin (data not shown).

To determine the TSH-induced changes at the level of the isoforms of CTF/NF1, we performed the EMSA with the labeled consensus CTF/NF1 oligonucleotide and nuclear extracts from hormone-deprived cells or treated 24 h with 1 nM TSH in the presence of two anti-CTF/NF1 antibodies. These were α-CTF/NF1/8199 antibody that recognize a N-terminal conserved sequence of all CTF/NF1 isoforms and an α-CTF/NF1/2902 antibody that immunoreacted specifically with the C-terminal peptide (amino acids 419–435) of the CTF/NF1-C protein. In the EMSA, the complexes formed with nuclear extracts from cells maintained in basal medium were recognized by the α-CTF/NF1/8199 antibody. This antibody reduced the intensity of the bands and at the same time generated two supershifts, one of which could barely enter the gel (Fig. 6, compare *lanes 2* and *3*). The slower migrating complexes obtained with extracts from cells treated with TSH were also recognized by the antibody forming complexes similar to that obtained with extracts from the uninduced cells. The only difference being that the supershifted complex that hardly entered the gel had a higher intensity (Fig. 6, compare *lanes 3* and *6*). This indicates a increase in the abundance of the CTF/NF1 proteins in this complex. To determine the presence of an increased CTF/NF1-C as suggested by the Northern blot experiments, we used the antibody α-CTF/NF1/2902 that specifically recognized this protein. With extracts from uninduced cells, only a weak supershifted band was observed indicating a low level of the CTF/NF1-C in the complexes (Fig. 6, *lane 4*). However, in extracts from TSH-treated cells, there was a shift to higher mobility complexes as reported in Fig. 5 which in the presence of αCTF/NF1/2902 generated a fairly strong supershifted band with a drastic reduction of the complexes 1 and 2 (Fig. 6, compare *lanes 5* and *7*). These results together demonstrate that TSH-treated FRTL-5 cells contain a lot more CTF/NF1-C protein compared with the non-treated cells and confirm our results in the Northern blot assay of TSH induced activation of CTF/NF1-C.

Physical Interaction between TTF-2 and Members of the CTF/NF1 Proteins—The close proximity of the CTF/NF1 and TTF-2 binding sites and inducibility of their expression suggested that they may physically cooperate in the hormonal regulation of expression at the TPO promoter. To investigate this idea, we examined the ability of *in vitro*-translated CTF/NF1 proteins to bind to a bacterially expressed GST-TTF-2 fusion protein and the effect of increasing the distance between these binding sites in transfection experiments.

In GST pull-down assay, radioactively labeled CTF/NF1-C, -X, or -B bound to the immobilized GST-TTF-2 but not GST alone (Fig. 7, compare *lanes 5, 9*, and *12* with *4, 8,* and *11*). A radioactively labeled LexA-VP16 fusion protein in a parallel reaction failed to bind to either GST or GST-TTF-2 (Fig. 7, *lanes 6* and *7*). This demonstrates the specificity of interaction of the CTF/NF1 proteins with TTF-2.

To determine whether alteration of the distance between these two factors can affect their function, we increased the spacing between their binding sites by 5 and 10 nucleotides p120 (+5) and p120 (+10) to generate half-helical and helical turns of the DNA helix (Fig. 8, *upper panel*). These constructs, together with the p420 and p120 TPO LUC as control, were transfected into the FRTL-5 cells and their activity determined in the absence and presence of TSH and insulin. Insertion of 5 bp (p120 (+5)TPO LUC) led to a reduction of the basal level of expression but this was restored by increasing the distance between the factors to 10 bp (Fig. 8*A*). Similar results were obtained after TSH and insulin treatment except that the response of the p120 (+10) construct to TSH was even higher than in the wild-type situation (Fig. 8*B*). The significance of this is not clear but would suggest that an increase in the distance of the two binding sites by 10 bp (one helical turn) may allow an even better interaction for transactivation than in the wild-type situation (Fig. 8*A*).

DISCUSSION

Thyroid-specific gene expression is achieved by a combination of multiple regulatory elements within the promoter of specific genes. One of the decisive genes that defines thyroid-specific function is the enzyme TPO. We have previously shown that the promoter element of this gene termed Z, where the thyroid-specific transcription factor TTF-2 binds, is the main mediator of the hormonal response of TPO transcription (16). Since TTF-2 activity depends on multimerization and specific orientation, we have characterized the B element at its 5′ adjacent region (see Fig. 1 for diagram) for possible cooperation with TTF-2. In this work we have shown that the B element is essential for the action of TTF-2 at the TPO promoter. We established this with the use of transfection experiments involving deletion and site-directed mutations of the B element in the TPO promoter. Our findings showed an important role for the hormone-regulated expression of a sequence in the B element which we identified as a binding site for CTF/NF1.

CTF/NF1 was originally identified as a host-encoded protein required for efficient initiation of adenovirus replication *in vitro* (53) and was later shown to function in the expression of several cellular genes (23, 54–57). CTF/NF1 can stimulate transcription by itself but binding sites for this factor are frequently found clustered with binding sites for other transcription factors such as AP-1, hepatocyte nuclear factor 3α, and steroid receptors (44, 58–62). Perhaps one of the most extensively studied effects of CTF/NF1 is that of modulating the action of steroid hormone receptors on expression at the MMTV promoter. In this example steroid hormone receptors

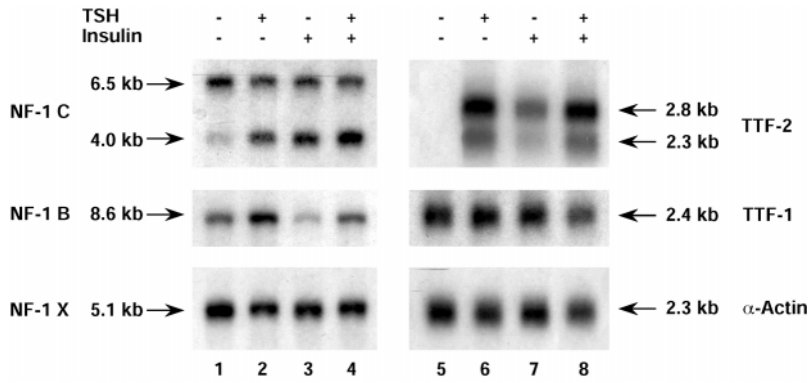

FIG. 4. **Hormonal regulation of mRNA levels of the transcription factors that bind to the *cis*-BZ regulatory region of the TPO promoter.** Poly(A)$^+$ RNA was extracted from FRTL-5 cells maintained 4 days in basal medium (*lanes 1* and *5*) or treated with 1 nM TSH (*lanes 2* and *6*), with 10 μg/ml insulin (*lanes 3* and *7*) or with both hormones together (*lanes 4* and *8*). The figure shows a representative Northern blot hybridized subsequently with CTF/NF1-C, CTF/NF1-B, CTF/NF1-X, TTF-2, TTF-1, and α-actin probes. The size of each transcript is indicated. The same results were obtained in three independent experiments.

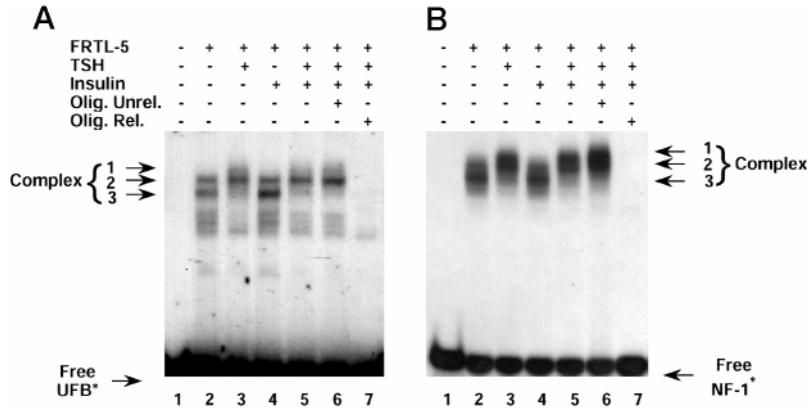

FIG. 5. **The CTF/NF1 binding activity is regulated by TSH.** EMSA performed with the labeled UFB oligonucleotide (*panel A*) or with the labeled CTF/NF1 consensus sequence (*panel B*) and 5 μg of nuclear extract from FRTL-5 cells maintained in absence of hormones (*lanes 2*), treated with TSH (*lanes 3*), with insulin (*lanes 4*), or with both hormones together (*lanes 5*). Competition experiments were performed on cells treated with both hormones using an unrelated (*lanes 6*) or a related oligonucleotide. The protein-DNA complexes are indicated by *arrows*.

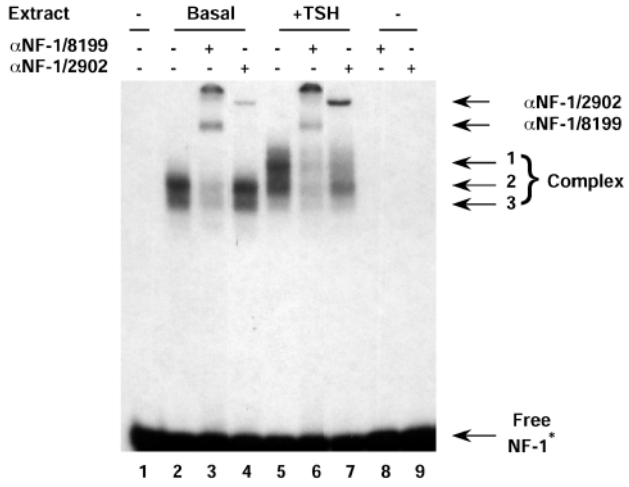

FIG. 6. **Identification of the TSH-induced DNA complex.** EMSA from nuclear extracts (5 μg) of FRTL-5 cells maintained in basal medium (*lane 2*) or treated 24 h with 1 nM TSH (*lane 5*) and the labeled CTF/NF1 consensus oligonucleotide. Supershift assays were performed in both basal and TSH-induced cells with the α-CTF/NF1/8199 antibody (*lanes 3* and *6*) or with α-CTF/NF1/2902 (*lanes 4* and *7*). For specificity of the supershift, antibodies were incubated with labeled oligonucleotides without nuclear extracts (*lanes 8* and *9*). The complexes found and the supershifts are indicated by *arrows*.

bind the nucleosomally organized promoter of MMTV and makes it accessible for CTF/NF1 to exert its transactivation effect (60, 63). The synthesis of CTF/NF1 is itself not affected by the steroid hormone. In our study we have shown that CTF/NF1 is not only involved in action of steroid hormone but also of thyroid hormones. This time it was not the action of a hormone receptor that was affected but rather the production of the hormone. As TPO is one of the key enzymes in the production of thyroid hormone, its regulation by CTF/NF1 is an example of a direct influence of this group of transcription factors on hormone production and action.

One important message of this paper is that the expression of CTF/NF1 is enhanced by TSH, via cAMP, and insulin. TSH increases the expression of CTF/NF1-B and -C while insulin up-regulates CTF/NF1-C but down-regulates CTF/NF1-B. This effect is specific as none of the inducers has any effect on CTF/NF1-X. In the case of TSH-induced expression of CTF/NF1-C we have further shown that this increase is associated with an enhanced synthesis of CTF/NF1-C. So far CTF/NF1 have been thought of as ubiquitously expressed genes. Recent studies have shown that certain CTF/NF1 genes are up-regulated during the metamorphic transition in *Xenopus laevis*. It is interesting to note that the two CTF/NF1 genes whose expression was enhanced by thyroid hormone and highly expressed during intestine remodeling turned out to be CTF/NF1-B and -C (64). These are the same genes whose expression we have identified as inducible by TSH and insulin. Analysis of the promoter regions of these genes will reveal how they are regulated by these hormones.

CTF/NF1 proteins bind DNA through a palindrome containing two TGGCA sequences on opposite strands. Furthermore,

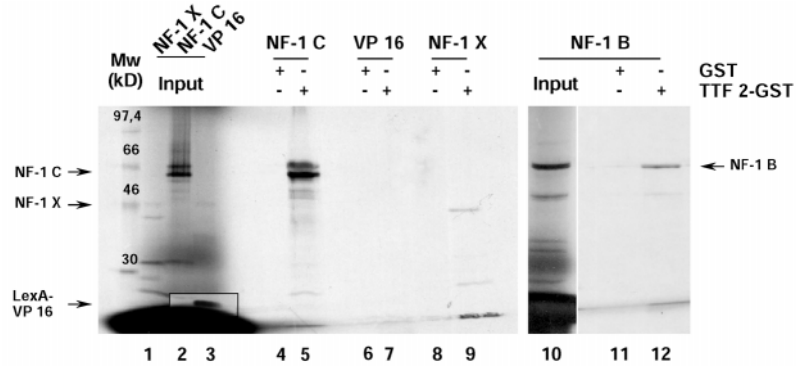

FIG. 7. **Physical interaction between transcription factors TTF-2 and CTF/NF1.** Pull-down assays were carried out with full-length TTF-2-GST fusion protein and with different members of the CTF/NF1 family. Lysate containing [^{35}S]methionine-labeled CTF/NF1-C, CTF/NF1-X, or CTF/NF1-B proteins was incubated with TTF-2-GST fusion protein (*lanes 5, 9,* and *12,* respectively) or GST (*lanes 4, 8*, and *11*, respectively) that had previously been coupled to glutathione-Sepharose beads and processed as described under "Experimental Procedures." The samples were resolved by SDS-PAGE analysis on a 10% polyacrylamide denaturing gel. Labeled *in vitro* translated Lex-A VP-16 protein was incubated under the same conditions with GST (*lane 6*) or TTF-2-GST (*lane 7*). Input (*lanes 1–3* and *10*) is reticulocyte lysate that had been programmed with the indicated templates.

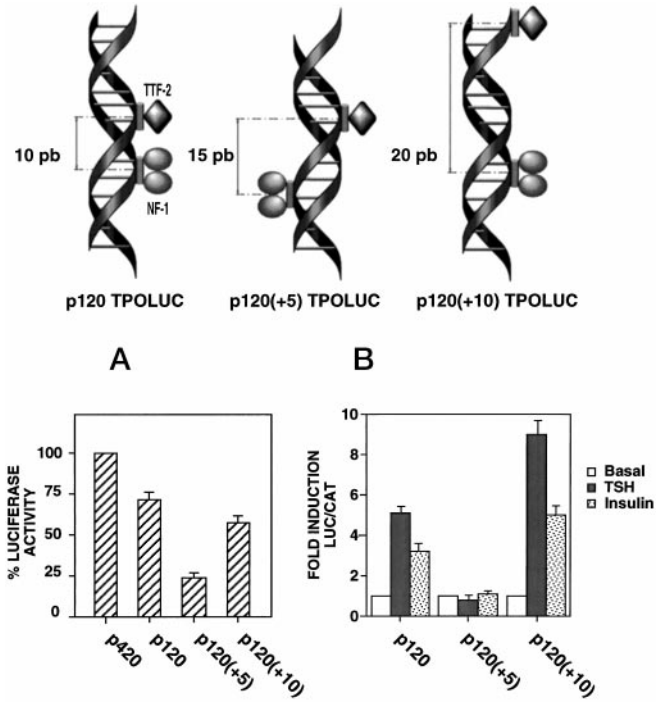

FIG. 8. **Increasing the distance between TTF-2 and NF-1 binding motifs impairs TPO promoter activity and the response to TSH and insulin is lost.** *Upper panel,* spatial model representing the DNA double helix of the TPO promoter. The distance between TTF-2 and CTF/NF-1-binding sites is maintained intact in the p120 construct, as in the wild type promoter, or is separated by an insertion of 5 (+5) or 10 (+10) bp, respectively, as described under "Experimental Procedures." *Lower panel,* TPO promoter activity derived from each construct transfected into FRTL-5 cells. *A,* luciferase activity was determined 48 h after the transfection in the proteins extracts from confluent cells maintained in complete medium. TPO promoter activity is expressed as light units normalized by transfection efficiency and is calculated relative to the activity of the wild type TPO promoter (p420 = 100%). The results are the mean ± S.D. of four different experiments. *B,* after transfection, cells were maintained 72 h in the absence of serum (0.2%) without TSH and insulin (basal medium). Then, the cells were treated with 1 nM TSH and 2 μM insulin for 24 h. Relative luciferase activity is the value of light units normalized by transfection efficiency. The TPO promoter activity is expressed as fold induction over the basal levels (= 1) of hormone-depleted cells. The results are the mean ± S.D. of four independent experiments.

several CTF/NF1 half-palindromes in many eukaryotic promoters are located close to other DNA element. Gil *et al.* (18) proposed that the binding of CTF/NF1 to such a sequence is stabilized by protein-protein interactions. In the TPO promoter the TTF-2 and the CTF/NF1-binding sites are separated by one turn of the DNA helix, suggesting that proteins bound to each element would be located on the same side of the DNA helix, in a position favorable for protein-protein contacts. Our results that a separation of these binding sites by a further 5 bp impaired TPO promoter activity but not by 10 bp, indicate that the stereospecific positioning of these elements is an important and necessary requirement for the transcriptional activity of the TPO promoter.

The synergistic interaction between CTF/NF1 and TTF-2 element may occur at the level of DNA binding or at the level of protein-protein interaction. Pull-down assays demonstrate that TTF-2 is able to interact physically with CTF/NF1 proteins. It is notable that CTF/NF1-X is not activated by TSH or insulin also interacted with TTF-2. Should these interactions occur *in vivo*, it will mean that physical association of the CTF/NF1 and TTF-2 is the main process of activating the TPO promoter activity. The hormone induced expression of CTF/NF1-C and -B therefore serves the main function of increasing the abundance of the CTF/NF1 proteins for this interaction. This suggests that the hormone enhanced expression of the CTF/NF1 transcription factors is to ensure the availability of enough CTF/NF1 proteins for interaction with TTF-2.

The interaction between CTF/NF1 and forkhead transcription factors could be a general mechanism of action of both families of transcription factors. A similar example has been reported in the modulation of liver-specific albumin transcription by HNF3-α (55). This idea is reinforced by the fact that another forkhead protein HNF3β is able to bind to the TTF-2-binding site (65). This conserved interaction between these families of transcription factors might be explained by their specific properties. Thus, the CTF/NF1-binding site is masked inside of the nucleosomal structure. The binding of the forkhead proteins to their cognate site desestabilizes this structure and makes the CTF/NF1 site accessible to exert its transactivation effect. The ability of the forkhead domain to induce DNA bending (66) would favor its contact with CTF/NF1 factors. This interaction could plays an important role in cell type-specific transcription and would be a widespread phenomenon that will be studied in the future.

Our data unequivocally demonstrate the importance of CTF/NFI transcription factors for thyroid-specific gene expression and show that some members of this family are hormonally inducible genes. Furthermore, CTF/NF1 factors interact *in vitro* with TTF-2, this interaction is functional and constitutes

a requirement for a correct hormonal response of the TPO promoter.

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The Interaction between the Forkhead Thyroid Transcription Factor TTF-2 and the Constitutive Factor CTF/NF-1 Is Required for Efficient Hormonal Regulation of the Thyroperoxidase Gene Transcription

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