

# Transcriptional Control of the Forkhead Thyroid Transcription Factor TTF-2 by Thyrotropin, Insulin, and Insulin-like Growth Factor I\*

(Received for publication, April 22, 1997, and in revised form, June 23, 1997)

Lourdes Ortiz‡, Mariastella Zannini§, Roberto Di Lauro¶, and Pilar Santisteban‡||

From the ‡Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, Arturo Duperier 4, 28029 Madrid, Spain, §Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università degli Studi di Napoli Federico II, via Pansini, 5, 80131 Naples, Italy, and ¶Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, Italy

**The hormonal regulation of both thyroglobulin and thyroperoxidase promoter activity in FRTL-5 thyroid cells takes place, at least in part, through a hormone-responsive element to which the thyroid transcription factor TTF-2 binds. The TTF-2 cDNA, encoded by the *tif2* locus, has recently been cloned and classified as a member of the forkhead transcription factor family. Here, we demonstrate that TTF-2 mRNA levels become undetectable in FRTL-5 thyroid cells cultured for 4 days in 0.2% serum and in the absence of thyrotropin (TSH) and insulin. Addition of TSH, insulin or insulin-like growth factor I (IGF-I) to the culture medium increases the levels of this transcription factor in a dose- and time-dependent manner and requires ongoing protein synthesis. The TSH effect is greater than that produced by insulin or IGF-I and is similar to the effect produced by the cAMP analog forskolin. The TSH and insulin effects are additive. In all cases, the mRNA level increase is accompanied by an increase in transcription rate, as demonstrated by run-off assays. These data demonstrate that the TTF-2 mRNA is under tight hormonal control. This is consistent with an important role for TTF-2 as a mediator of the transcriptional activation of thyroid-specific genes (thyroglobulin and thyroperoxidase) by TSH via cAMP and by insulin through the IGF-I receptor.**

Thyroid hormone biosynthesis takes place exclusively in the thyroid gland by iodinating and coupling the tyrosine residues in thyroglobulin (Tg)<sup>1</sup> by the enzyme thyroperoxidase (TPO) (EC 1.11.1.7.) (1–3). Expression of the Tg and TPO genes is restricted to thyroid tissue (4) and is under hormonal control. The pituitary hormone TSH, via cAMP, insulin, and insulin-like growth factor I (IGF-I) exerts a positive effect on Tg (5–9)

\* This work was supported by Grants DGICYT (PB94–0092), CAM (AE00310/95) (Spain), the Commission of European Communities (BIO2 CT 930454) and by grants from the Progetto Finalizzato Applicazioni Cliniche della Ricerca Oncologica of Consiglio Nazionale delle Ricerche, the Associazione Italiana per la Ricerca sul Cancro (Italy). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

|| To whom correspondence should be addressed: Instituto de Investigaciones Biomédicas, C/Arturo Duperier 4, Madrid 28029, Spain. Tel.: 34-1-5854644; Fax: 34-1-5854587; E-mail: psantisteban@biomed.iib.uam.es.

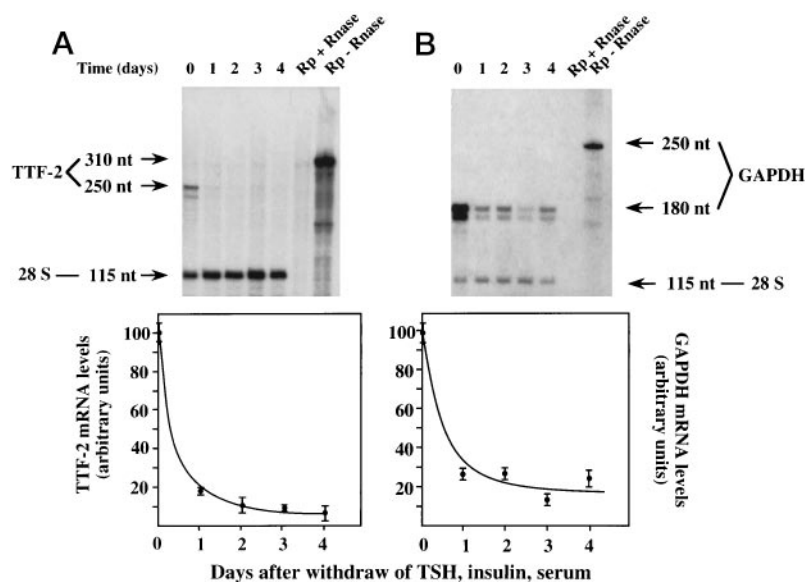
<sup>1</sup> The abbreviations used are: Tg, thyroglobulin; TPO, thyroperoxidase; IGF-I, insulin-like growth factor I; CHX, cycloheximide; GADPH, glyceraldehyde-3-phosphate dehydrogenase; nt, nucleotide(s); CRE, cAMP response element; IRE, insulin response element.

and TPO (10–14) gene expression. The hormonal regulation mechanism of Tg and TPO promoter activities has been studied extensively in the last few years, since their promoters are well characterized (15, 16). Three tissue-specific transcription factors, TTF-1, TTF-2, and Pax-8, have been shown to bind to both Tg and TPO promoters (4); they have been classified into three classes according to the structural motifs used for recognition-specific DNA sequences (17). Transient transfection experiments have demonstrated that the minimal Tg and TPO promoters that confer thyroid-specific expression also confer responsiveness to TSH (18–23), insulin, and IGF-I (18, 19, 23). TTF-1 and Pax-8, homeobox and paired box genes, respectively, were the first thyroid transcription factors cloned (24, 25). As a result, their possible role in hormonal regulation of Tg and TPO gene transcription has been studied extensively, although no clear role for these factors has been established (19, 26, 27).

We have previously demonstrated that the hormonal regulation of both Tg and TPO promoter activities in FRTL-5 cells takes place mainly through the *cis*-regulatory element, to which the thyroid transcription factor TTF-2 binds (19, 23). This factor binds to a single site in both promoters, and this binding site acts as a hormone-responsive element (23). The TTF-2 cDNA has recently been cloned and shown to be transcribed from a locus, denominated *tif2*, on mouse chromosome 4 (28). The TTF-2 protein is a new member of the forkhead family of transcription factors, a large and growing family whose members bind to DNA as monomers and contain a common 100-amino acid DNA-binding domain (29–32). This family of factors, also called FREAC or HNF-3 (hepatocyte nuclear factor 3) has been implicated in pattern formation during embryogenesis. TTF-2 binds to Tg and TPO also as a monomer (15, 16), and its expression, both during development and in adult tissue, is restricted to the thyroid and anterior pituitary and appears as early as day 8.5 of mouse gestation (28).

We analyze here whether the previously observed hormonal regulation of TTF-2-DNA complex formation (19, 23) correlates with an increase in TTF-2 mRNA. RNase protection assays were performed with total RNA from FRTL-5 cells, cultured either in the absence or presence of different hormones, and a riboprobe corresponding to the 3′-noncoding region of TTF-2 cDNA (28). The results indicated that TTF-2 mRNA is undetectable in FRTL-5 cells maintained for 4 days in 0.2% serum and in the absence of TSH and insulin. The addition of TSH, insulin, or IGF-I to the culture medium induced TTF-2 mRNA levels in a dose- and time-dependent manner and required ongoing protein synthesis. The TSH effect was greater than that produced by insulin or IGF-I and was mimicked by the cAMP analog forskolin. Moreover, the TSH effect was additive

**FIG. 1. Decreased TTF-2 mRNA levels in FRTL-5 cells deprived of TSH, insulin, and serum.** Confluent FRTL-5 cells were shifted to basal medium (no TSH, no insulin, and 0.2% serum). At the times noted (0, 1, 2, 3, and 4 days) total RNA was isolated. *Upper panels*, representative gel of an RNase protection assay showing a 250-nt fragment of the TTF-2 mRNA (*panel A*) or a 180-nt fragment of GAPDH mRNA (*panel B*). A 115-nt fragment of 28 S ribosomal RNA was used in all the experiments to correct for total RNA loading. In both *panels*, the radioactive riboprobe (310 nt for TTF-2 or 250 nt for GAPDH) plus or minus RNase, was used. *Lower panel*, quantitation of mRNA by densitometric gel scanning. Each value represents the mean  $\pm$  S.D. of three independent experiments.



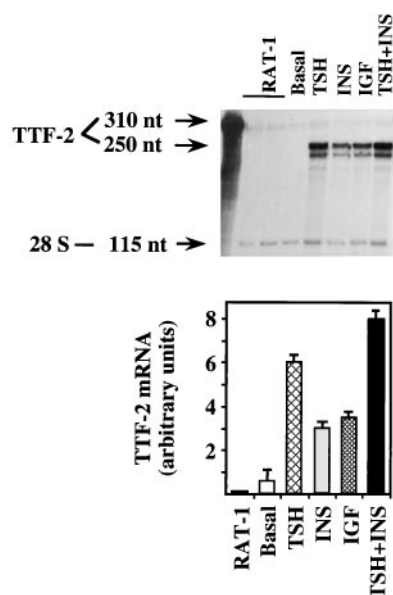
to those of insulin or IGF-I. Run-off assays indicate that the hormone-induced increases in mRNA levels are accompanied by a transcription rate increase. These data indicate that the levels of mRNA encoding TTF-2 are tightly controlled by TSH/cAMP and by insulin/IGF-1 and support the idea of an important role for the TTF-2 transcription factor in the hormonal regulation of Tg and TPO gene expression.

#### EXPERIMENTAL PROCEDURES

**Materials**—Tissue culture medium, bovine TSH, bovine insulin, and cycloheximide (CHX) were purchased from Sigma and forskolin from Boehringer Mannheim. Rat IGF-1 was from Amgen Biologicals (Thousand Oaks, CA). Donor, fetal calf serum, and DMEM were from Life Technologies, Inc.; nylon membranes were obtained from Bio-Rad. pT7RNA-28S and the RPA II assay kit were purchased from Ambion (Austin, TX), the Riboprobe Transcription kit from Promega (Madison, WI), and [ $\alpha$ - $^{32}$ P]rUTP or [ $\alpha$ - $^{32}$ P]rCTP from ICN (Irvine, CA).

**Cell Culture**—FRTL-5 cells (ATTC CRL 8305; American Type Culture Collection) were cultured as described previously (33) in Coon's modified Ham's F-12 medium supplemented with 5% donor calf serum and with a six-hormone mixture including 1 nM TSH and 10  $\mu$ g/ml insulin (complete medium). The effect of hormones and growth factor were studied by starving confluent cells for TSH and insulin in the presence of 0.2% serum (basal medium). After 4 days each ligand was added to the culture medium at the concentrations noted. In some experiments cells were treated with CHX, at a concentration of 10  $\mu$ g/ml for 24 h. In this conditions, more than 95% protein synthesis was inhibited. Rat-1 fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum.

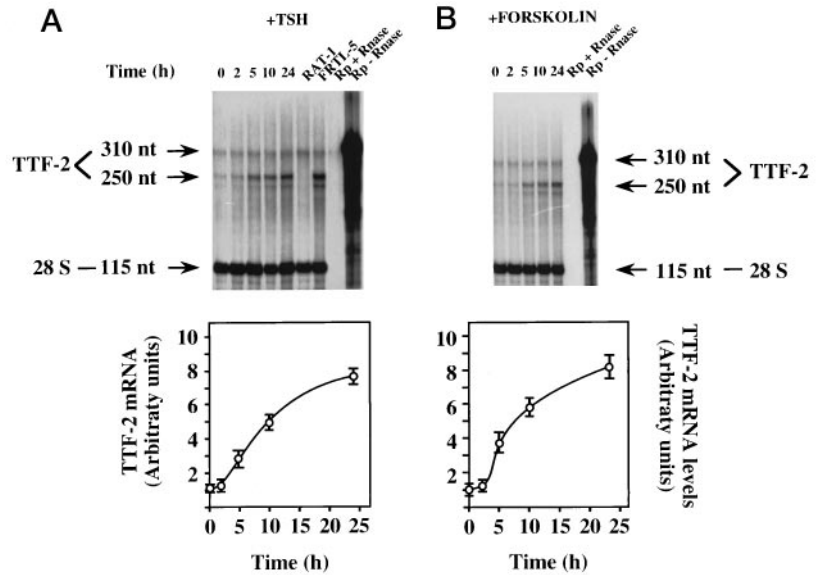
**Ribonuclease Protection Assay**—Total cell RNA was extracted by the guanidinium isothiocyanate method (34). A 250-base pair fragment of the 3'-noncoding region of TTF-2 cDNA was cloned into the *EcoRV* site of the pBS SK<sup>+</sup> vector. pBS-GAPDH was generated by cloning a 189-base pair fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (35) into the *EcoRI* site of the pBS SK<sup>+</sup> vector.  $^{32}$ P-labeled antisense riboprobes were generated using T<sub>7</sub> RNA polymerase, [ $\alpha$ - $^{32}$ P]CTP (800 Ci/mmol) and the Riboprobe Transcription kit according to the manufacturer's instructions. As an internal control to correct for total RNA loading, an additional 28 S ribosomal antisense riboprobe was transcribed from pT7RNA-28S. The mRNA levels were analyzed by RNase protection with the RPA II assay kit, essentially as described by the manufacturer. TTF-2 or GAPDH ( $5 \times 10^5$  cpm) and  $5 \times 10^4$  cpm of 28 S riboprobes were incubated overnight with 15  $\mu$ g of total RNA. After hybridization, samples were digested with an RNase mixture (2.5 units/ml RNase A and 50 units/ml T1 ribonuclease) for 2 h at 37 °C. The protected fragments were precipitated and electrophoresed on a denaturing 5% acrylamide gel, dried, and visualized by autoradiography. The mRNA levels and ribosomal 28 S RNA were quantified by densitometric gel scanning in an Instantimager (Packard, Meriden, CT) and the relative TTF-2 or GAPDH mRNA levels expressed in arbitrary units after correction with the 28 S ribosomal RNA levels.



**FIG. 2. TSH, insulin, and IGF-I up-regulate TTF-2 mRNA levels.** Total RNA from FRTL-5 cells maintained for 4 days in basal medium or treated for 24 h with 1 nM TSH, 10  $\mu$ g/ml insulin, 100 ng/ml IGF-I, or TSH plus insulin was isolated. Total RNA from a non-thyroid cell line, the fibroblast Rat-1, was used in parallel. *Upper panel*, representative RNase protection assay showing the 250-nt fragment of TTF-2 mRNA and the 115-nt fragment of 28 S ribosomal RNA. The *first lane* of the gel is the 310-nt fragment of TTF-2 riboprobe without RNase. *Lower panel*, quantitation of mRNA by densitometric gel scanning. Each value represents the mean  $\pm$  S.D. of three independent experiments.

**Run-off Assays**—Nuclear isolation and run-off transcription labeling were performed according to the method described by Greenberg *et al.* (36). Nuclei were incubated with [ $\alpha$ - $^{32}$ P]UTP (800 Ci/mmol) to label nascent RNA transcripts. Newly transcribed RNA was extracted by the guanidinium isothiocyanate method (34). Plasmid DNA (10  $\mu$ g) was linearized and denatured prior to slot blotting onto a nylon membrane and UV crosslinking. Each filter contained TTF-2 and GAPDH cDNA plasmids and two additional plasmids:  $\beta$ -actin as a control gene not hormonally regulated in this system (8) and pBS SK<sup>+</sup> as the control for nonspecific hybridization. Filters were hybridized in 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2, 1 mM EDTA, pH 8.0, 7% SDS (w/v), 45% formamide (v/v), and 250  $\mu$ g/ml tRNA carrier and equal amounts of radioactivity of the appropriate experimental groups. Hybridization was performed for 48 h at 42 °C and filters washed in 40 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2, and 1% SDS (w/v) for 15 min each at successively higher temperatures, room temperature, 37 °C and 55 °C. Newly transcribed RNA levels were deter-

**FIG. 3. Time-dependent TTF-2 up-regulation by TSH and forskolin.** FRTL-5 cells cultured for 4 days in basal medium (time 0) were treated with 1 nM TSH (A) or 10  $\mu$ M forskolin (B) for 2, 5, 10, and 24 h. In panel A, Rat-1 cells were used as a control with no expression of TTF-2 mRNA, and confluent FRTL-5 cells as a control of maximal expression. Upper panels, representative RNase protection assay showing the 250-nt fragment of TTF-2 mRNA and the 115-nt fragment of the 28 S ribosomal RNA. In both panels, the last two lanes are the 310-nt fragment of TTF-2 riboprobe plus or minus RNase. Lower panel, quantitation of 250-nt band by densitometric gel scanning. Each value represents the mean  $\pm$  S.D. of three independent experiments.

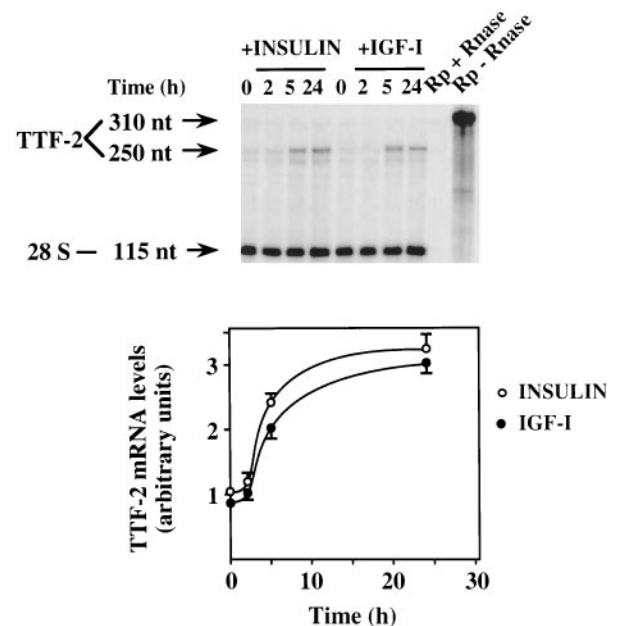


mined by autoradiography and quantitated by densitometric scanning of the blots in an Instantimager.

#### RESULTS

**TTF-2 Gene Expression Is Virtually Absent in FRTL-5 Thyroid Cells Cultured in Hormone- and Serum-free Medium**—Previous results from our laboratory (19, 23) showed that TTF-2 DNA binding activity cannot be detected in FRTL-5 cells cultured for 4 days in 0.2% serum and in the absence of TSH and insulin (basal medium). When TSH or insulin is added, TTF-2 DNA binding activity is restored to normal levels. These effects could be explained by a tight hormonal control of either the DNA binding activity or the synthesis of TTF-2. The recent cloning of TTF-2 cDNA (28) permitted direct testing of the latter hypothesis. For this, confluent FRTL-5 cells were maintained in basal medium for 1, 2, 3, and 4 days. Total RNA from each group was isolated, and TTF-2 mRNA levels were determined by the RNase protection assay. The RNA probe used is 310 nucleotide (nt) long and contains, in addition to 60 nt of vector sequences, 250 nt complementary to the 3'-untranslated region of TTF-2 mRNA (28). A major, 250-nt-long protected RNA fragment was detected in confluent FRTL-5 cells (Fig. 1A, time 0). Other fragments, including one of about 245 nt, were also detected, presumably originated from spurious cleavage by RNase. The most abundant 250-nt fragment was used in all densitometric analysis reported here, since we observed that all fragments are identically regulated. After 1 day without hormones, TTF-2 mRNA levels had already dropped to 20% of their maximal expression. After 4 days, TTF-2 expression levels were only 10% of the maximum. Experiments involving readdition of the ligands were performed after 4 days without hormones.

GAPDH gene expression, reported to be under insulin regulation (35, 37), was used as a positive control; GAPDH mRNA levels were also determined in the RNase protection assay with total RNA from the same samples used above. A major 180-nt GAPDH mRNA fragment was protected in confluent FRTL-5 cells (Fig. 1B, time 0). GAPDH expression in cells maintained in 0.2% serum and in the absence of insulin decreased, as expected, to approximately 20% of the levels observed in confluent cells. In both TTF-2 and GAPDH RNase protection assays, a 115-nt fragment of the 28 S ribosomal RNA was used as a control for the quality and quantity of RNA used in the protection experiments. The lower panels represent the scanner densitometry from three independent experiments. RNA



**FIG. 4. Time-dependent TTF-2 up-regulation by insulin and IGF-I.** FRTL-5 cells maintained 4 days in basal medium (time 0) were treated with 10  $\mu$ g/ml insulin or 100 ng/ml IGF-I for 2, 5, 10, and 24 h. Upper panel, representative RNase protection assay showing the 250-nt fragment of TTF-2 mRNA and the 115-nt fragment of 28 S ribosomal RNA. The last two lanes are the 310-nt fragment of TTF-2 riboprobe plus or minus RNase. Lower panel, quantitation of mRNAs by densitometric gel scanning. Each value represents the mean  $\pm$  S.D. of three independent experiments.

levels are expressed relative to the levels obtained in confluent cells, arbitrarily set at 100%.

**TSH via cAMP, Insulin, and IGF-I Increases TTF-2 mRNA Levels**—FRTL-5 cells maintained in basal medium for 4 days were treated with 1 nM TSH, 10  $\mu$ g/ml insulin, 100 ng/ml IGF-I or TSH plus insulin for 24 h. Total RNA was isolated and an RNase protection assay performed. TTF-2 mRNA was undetectable in FRTL-5 cells maintained in basal medium, as well as in Rat-1 fibroblasts (Fig. 2, top panel). Readdition of TSH, insulin or IGF-I at the concentrations indicated stimulated TTF-2 mRNA levels within 24 h. Scanning densitometry of three separate experiments show that the TSH effect is greater (7-fold) than that induced by insulin or IGF-I (3- to 4-fold). The

effect of TSH and insulin was additive. Hormone and growth factor concentrations used were deduced by dose-response experiments (data not shown) and were the optimum values used in the FRTL-5 cells culture medium (33).

The increase in TTF-2 mRNA levels was already detectable within five hours after the addition of 1 nM TSH (Fig. 3A), 10 μg/ml insulin, or 100 ng/ml IGF-I (Fig. 4). Longer incubations (up to 24 h) resulted in a progressive signal increase. The cAMP analog forskolin, at the concentration of 10 μM, elicited a similar time course pattern of TTF-2 mRNA expression than that

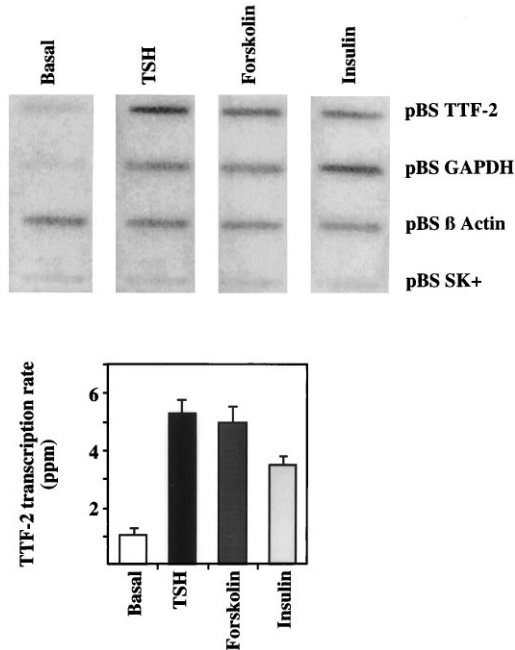


FIG. 5. TTF-2 mRNA gene transcription rate in response to various hormones. Nuclei were isolated from FRTL-5 cells maintained for 4 days in basal medium or treated for 24 h with 10 nM TSH, 10 μM forskolin, or 10 μg/ml insulin. Approximately 30 × 10<sup>6</sup> nuclei from each sample were subjected to nuclear run-off analysis. The newly synthesized RNA was isolated and hybridized to immobilized cDNAs (TTF-2, GAPDH, β-actin) and the pBS SK<sup>+</sup> plasmid on nylon membranes. Blots were exposed for 1 week. *Upper panel*, autoradiogram from a representative experiment. *Lower panel*, band intensity after scanning densitometry of blots. Values were corrected for nonspecific binding by subtracting the hybridization signal of the vector alone (pBSK<sup>+</sup>), and results were normalized using β-actin values. Each value represents the mean ± S.D. of three independent experiments.

exerted by TSH (Fig. 3B). It is important to note that the above kinetics of TTF-2 mRNA are slow and always consistent with the changes in TTF-2 binding activity previously reported (19, 23).

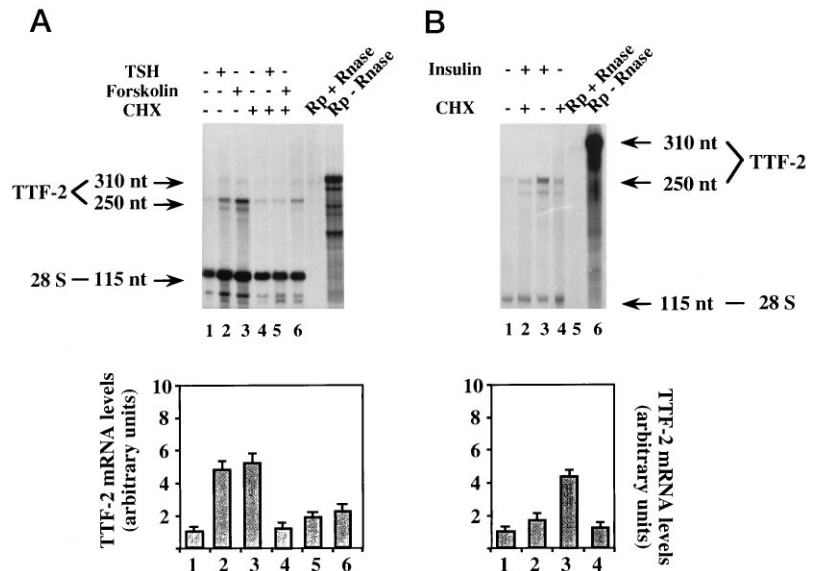
**TSH and Insulin Stimulate TTF-2 Gene Transcription**—To determine whether hormone treatment increases TTF-2 mRNA synthesis, run-off assays were performed with nuclei isolated from cells maintained in basal medium or treated for 24 h with 1 nM TSH, 10 μM forskolin, and 10 μg/ml insulin (Fig. 5). These three ligands induced a variable amount of newly transcribed TTF-2 mRNA in three independent experiments. The increase caused by TSH and forskolin was similar (approximately 5-fold) and greater than that induced by insulin (approximately 3-fold). Considering that β-actin transcription in FRTL-5 cells is not regulated by the above hormones (8), the percentage of increase was calculated relative to this gene. In all experiments, slots containing the GAPDH plasmid were used as a positive control of insulin induction of gene transcription. It is interesting to note that, in 0.2% serum and in the absence of insulin, TSH, and forskolin also induced GAPDH gene transcription. When cells maintained in basal conditions were incubated for 24 h in complete medium (5% serum), the transcription rate of TTF-2 and GAPDH returned to normal levels (data not shown). The pBS SK<sup>+</sup> plasmid was used throughout as a negative control.

**TSH and Insulin Induction of TTF-2 Gene Expression Requires Ongoing Protein Synthesis**—Total RNA was extracted from FRTL-5 cells maintained in basal medium or treated for 24 h with TSH, forskolin or insulin alone or in combination with 10 μg/ml CHX. After RNase protection assay, TTF-2 mRNA levels were determined. TSH and forskolin increased the TTF-2 mRNA levels (Fig. 6A, lanes 2 and 3). CHX alone does not significantly induce TTF-2 mRNA (lane 4) but blocks the increase induced by TSH and forskolin (lanes 5 and 6). The same results were obtained in panel B when insulin (lane 3), CHX (lane 4), or both substances together (lane 2) were used. This suggests that *de novo* protein synthesis is involved in the mechanism of TTF-2 gene induction by these ligands.

DISCUSSION

We previously reported that similar *cis*-regulatory elements, recognized by the TTF-2 DNA binding activity, are responsible for Tg and TPO promoter activity regulation by TSH and insulin (19, 23). We also demonstrated that TTF-2 binding to Tg and TPO promoter is under hormonal regulation in a time- and

FIG. 6. CHX inhibits the TSH, forskolin, or insulin induction of TTF-2 mRNA levels. FRTL-5 cells were cultured in basal medium for 4 days. At this time, 10 nM TSH (panel A), 10 μM forskolin (panel A), 10 μg/ml insulin (panel B), or 5 μg/ml CHX were added alone or in combination for 24 h and total RNA isolated. *Upper panels*, representative RNase protection assays showing the 250-nt fragment of TTF-2 mRNA and the 115-nt fragment of the 28 S ribosomal RNA. The last two lanes in both panels are the 310-nt fragment of TTF-2 riboprobe plus or minus RNase. *Lower panels*, quantitation of mRNA by densitometric gel scanning from three independent experiments.



dose-dependent manner (19, 23). The effect of TSH was mimicked by forskolin, and high doses of insulin produced the same promoter stimulation as low doses of IGF-I. On the basis of these data, we suggested that signals originated at either the TSH or the IGF-I receptors modulate Tg and TPO gene transcription by regulating the levels of TTF-2 DNA binding activity. The above experiments did not provide direct evidence, however, as to whether the hormones regulate the binding activity or the synthesis of TTF-2 directly. The recent cloning of TTF-2 cDNA (28) permitted us to distinguish between these possibilities. TTF-2 is a member of the forkhead/HNF-3 family of transcription factors (29–32), and, among adult rat tissues, its expression has been detected exclusively in thyroid tissue (28). TTF-2 is expressed transiently in the thyroid (and anterior pituitary) bud and its mRNA is down-regulated just before the onset of thyroid differentiation; in adult thyroid tissue TTF-2 mRNA is detected again, indicating a complex temporal regulation operating on TTF-2 mRNA levels (28). To define the mechanisms responsible for the hormonal regulation of TTF-2, we determined TTF-2 mRNA levels in FRTL-5 cells maintained in 0.2% serum and in the presence or absence of various hormones. The results demonstrated that physiological doses of TSH and high doses of insulin increases TTF-2 mRNA levels. This regulation is specific since under the same treatment condition, we have previously reported that the levels of  $\beta$ -actin mRNA remain unaltered (8). The same effect on TTF-2 was obtained with forskolin and low doses of IGF-I, suggesting that TSH acts through the cAMP pathway and insulin mainly through the IGF-I receptor to modulate the concentration of the transcription factor in thyroid cells. These data are in keeping with the previously described hormonal regulation of Tg and TPO genes (8, 19, 23), providing further evidence for a role for TTF-2 in mediating this regulation.

The above ligands increase TTF-2 mRNA synthesis, indicating that the hormonal control of *tif2* gene expression operates at transcriptional level. It is important to mention that this is the first evidence of a specific transcription factor regulated by insulin at the transcriptional levels. However, we have not ruled out the possibility that postranslational mechanisms may also be involved. The hormonal induction of TTF-2 mRNA levels required ongoing protein synthesis, as we previously demonstrated for the hormonal regulation of TTF-2 binding (18, 23), suggesting that the *tif2* gene is in turn controlled by factors whose synthesis is hormonally regulated. Alternatively, the requirement for protein synthesis to obtain maximal TTF-2 induction could indicate the operation of an autoregulatory mechanism.

Tg and TPO promoters constitute an unusual example in which insulin and cAMP increase their transcription. As both pathways normally exert an antagonistic effect (37), we expected to find cAMP and insulin response elements (CRE and IRE) within the Tg and TPO promoter sequences. Strikingly, no consensus CRE (38, 39) or any of the IRE (37) defined so far have been identified. A CRE-like (40) and a CRE-enhancer (41) have been described in the dog and human Tg promoter, although these elements only share partial homology with the canonical CRE palindrome. These observations suggest that Tg and TPO transcription control by cAMP and insulin involves a distinct mechanism. It may be hypothesized that this circumstance could be exclusive to thyroid genes; however, the presence of a CRE and an IRE element within the TSH-R promoter (42, 43) rules out this possibility. Further studies are necessary to determine whether TTF-2 is the final target of insulin and TSH action through independent pathways or there is cross-talk in the response. In this context, it is important to determine whether the *tif2* gene promoter contains a CRE and an

IRE, or is itself regulated by another protein. The observation that thyroid cells contain the transcription factor NGFI-A, whose synthesis is rapidly induced by cAMP (44) is of great interest as will be the determination of the relative role of TTF-2 and NGFI-A in controlling cAMP-regulated transcription in thyroid. Other thyroid-specific transcription factors, such as TTF-1 and Pax-8, have been suggested to be under TSH/cAMP control (27, 42), although none show the fine and clear TSH/cAMP regulation presented here for TTF-2. The hormonal regulation of both TTF-1 and Pax-8 mRNA remains a matter of controversy. Down-regulation of TTF-1 mRNA levels after a few hours of TSH treatment has been described for FRTL-5 cells (42), but no such regulation was observed in cultured dog thyrocytes (26). Our previous data have shown that TTF-1 mRNA is not regulated by insulin, IGF-I (19) or by TSH (45). It has been reported that TSH also regulates TTF-1 and Pax-8 binding to DNA by a redox mechanism (46). These results are interesting and compatible with the one obtained here, demonstrating that the transcription factor TTF-2 plays a major role in Tg and TPO gene expression, but is presumably not the only factor involved.

The fact that TTF-2 is a transcription factor with a forkhead domain makes its hormonal regulation very interesting. The forkhead/HNF-3 transcription factor family members share a core consensus binding sequence, RTAAAYA, but differ in the positions flanking the core, creating differences in DNA binding specificity. The core consensus binding sequence in Tg (AGAAAAC) and TPO (CTAAACA) could differ sufficiently to explain some of the differences in the regulation of the two thyroid genes. The binding of forkhead proteins to their cognate site results in bending of DNA at an 80–90° angle (29); this property could be decisive for the appropriate spatial interaction between TTF-2 and other transcription factors within the Tg and TPO promoters. These other factors, although similar in both promoters, are not identical and are not located at the same distance from the TTF-2 binding site. The interaction between TTF-2 and other transcription factors, thyroid-specific or ubiquitous, will thus be slightly different, allowing a different extent of transcription and perhaps distinct regulation of each gene. HNF-3 $\beta$ , another forkhead family member, binds at the same position and to the same sequence as TTF-2 in the TPO promoter but not in the Tg promoter (47). HNF-3 $\beta$  and TTF-2 thus show functional redundancy, as they both appear to stimulate transcription through the same DNA sequence. It will be of interest to determine whether the HNF-3 $\beta$  gene is regulated by the same factors as TTF-2.

*Acknowledgments*—We thank Dr. Domingo Baretino for discussions and Dr. Juan A. Velasco and Dr. Erik Wade for the critical reading of this manuscript.

#### REFERENCES

1. Taurog, A. (1976) in *The Thyroid: A Fundamental and Clinical Text* (Braverman, L. E., and Utiger, R. D., eds) pp. 47–81, Lippincott-Raven, New York
2. De Groot, L. J., and Niepomniszcze, H. (1977) *Metabolism* **26**, 665–718
3. Salvatore, G., Stambury, J. B., and Rall, J. E. (1980) in *The Thyroid Gland* (De Visscher, M., ed) pp. 443–487, Raven Press, New York
4. Damante, G., and Di Lauro, R. (1994) *Biochim. Biophys. Acta* **1218**, 255–266
5. Van Heuverswyn, G., Streydio, C., Brocas, H., Refetoff, S., Dumont, J., and Vassart G. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 5941–5945
6. Avvedimento, V. E., Tramontano, D., Ursini, M. V., Monticelli, A., and Di Lauro, R. (1984) *Biochem. Biophys. Res. Commun.* **122**, 472–477
7. Van Heuverswyn, G., Leriche, A., Van Sande, J., Dumont, J., and Vassart, G. (1985) *FEBS Lett.* **188**, 192–196
8. Santisteban, P., Kohn, L. D., and Di Lauro, R. (1987) *J. Biol. Chem.* **262**, 4048–4052
9. Chabaud, O., Chambard, M., Gaudry, N., and Mauchamp, J. (1988) *J. Endocrinol.* **116**, 25–33
10. Gérard, C. M., Lefort, A., Chirtophe, D., Libert, F., Van Sande, J., Dumont, J. E., and Vassart G. (1988) *Mol. Endocrinol.* **3**, 2110–2118
11. Iozaki, O., Kohn, L. D., Kozak, C. A., and Kimura, S. (1989) *Mol. Endocrinol.* **3**, 1681–1692
12. Gerard, C. M., Lefort, A., Libert, F., Christophe, D., Dumont J. E., and Vassart,

- G. (1988) *Mol. Cell. Endocrinol.* **60**, 239–242
13. Damante, G., Chazenbalk, G., Russo, D., Rapoport, B., Foti, D., and Filetti, S. (1989) *Endocrinology* **124**, 2889–2894
  14. Zarrilli, R., Formisano, S., and Di Jeso, B. (1990) *Mol. Endocrinol.* **4**, 39–45
  15. Civitarreale, D., Lonigro, R., Sinclair, A., and Di Lauro, R. (1989) *EMBO J.* **8**, 2537–2542
  16. Francis-Lang, H., Price, M., Polycarpou-Schwarz, M., and Di Lauro, R. (1992) *Mol. Cell. Biol.* **12**, 576–588
  17. Pabo, C. O., and Sauer, R. T. (1992) *Annu. Rev. Biochem.* **61**, 1053–1095
  18. Lee, N. T., Nayfeh, S. N., and Chae, C. B. (1988) *J. Biol. Chem.* **264**, 7523–7530
  19. Santisteban, P., Acebrón, A., Polycarpou-Schwarz, M., and Di Lauro, R. (1992) *Mol. Endocrinol.* **6**, 1310–1317
  20. Skinner, C. A., Kelly, D., Kao, L. R., Nayfeh, S. N., Popko, B., and Chae, C. B. (1992) *Mol. Cell. Endocrinol.* **90**, 33–38
  21. Abramowicz, M. J., Vassart, G., and Christophe, D. (1990) *Biochem. Biophys. Res. Commun.* **166**, 1257–1264
  22. Abramowicz, M. J., Vassart, G., and Christophe, D. (1992) *Eur. J. Biochem.* **203**, 467–473
  23. Aza-Blanc, P., Di Lauro, R., and Santisteban, P. (1993) *Mol. Endocrinol.* **7**, 1297–1306
  24. Guazzi, S., Price, M., De Felice, M., Damante, G., Mattei, M. G., and Di Lauro, R. (1990) *EMBO J.* **9**, 3631–3639
  25. Zannini, M. E., Francis-Lang, H., Plachov, D., and Di Lauro, R. (1992) *Mol. Cell. Biol.* **12**, 4230–4231
  26. Van Renterghem, P., Dremier, S., Vassart, G., and Christophe, D. (1995) *Mol. Cell. Endocrinol.* **112**, 83–93
  27. Van Renterghem, P., Vassart, G., and Christophe, D. (1996) *Biochim. Biophys. Acta* **1307**, 97–103
  28. Zannini, M., Avantaggiato, V., Biffali, E., Arnone, M. I., Sato, K., Pischetola, M., Taylor, B. A., Phillips, S. J., Simeone, A., and Di Lauro, R. (1997) *EMBO J.* **16**, 3185–3197
  29. Pierrou, S., Hellqvist, M., Samuelson, L., Enerbäck, S., and Carlsson, P. (1994) *EMBO J.* **13**, 5002–5012
  30. Lai, E., Clark, K. L., Burley, S. K., and Darnell, J. E., Jr. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10421–10423
  31. Kästner, K. H., Hiemisch, H., Luckow, B., and Schütz, G. (1994) *Genomics* **20**, 377–385
  32. Pani, L., Ovendier, D. G., Porcella, A., Qian, X., Lai, E., and Costa, R. H. (1992) *Mol. Cell. Biol.* **12**, 3723–3732
  33. Ambesi-Impiombato, F. S., Parks, L. A. M., and Coe, H. G. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 3455–3459
  34. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
  35. Alexander, M. C., Lomanto, M., Nasrin, N., and Ramaika, C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5273–5277
  36. Greenberg, M. E., and Bender, T. P. (1994) *Curr. Protein Mol. Biol. Suppl.* **26**
  37. O'Brien, R. M., and Granner, D. K. (1996) *Physiol. Rev.* **76**, 1109–1161
  38. Montminy, M. R., Sevario, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 6682–6686
  39. Habener, J. F. (1990) *Mol. Endocrinol.* **4**, 1087–1094
  40. Christophe, D., Gérard, C., Juvenal, G., Bacolla, A., Teugels, E., Ledent, C., Christophe-Hobertus, C., Dumont, J. E., and Vassart, G. (1989) *Mol. Cell. Endocrinol.* **64**, 5–18
  41. Berg, V., Vassart, G., and Christophe, D. (1996) *Biochim. Biophys. Acta* **1307**, 35–38
  42. Shimura, H., Okajima, F., Ikumaya, S., Shimura, Y., Kimura, S., Saji, M., and Kohn, L. D. (1994) *Mol. Endocrinol.* **8**, 1049–1069
  43. Shimura, Y., Shimura, H., Ohmori, M., Ikuyama, S., and Kohn, L. D. (1994) *J. Biol. Chem.* **269**, 31908–31914
  44. Pichon, B., Jiménez-Cervantes, C., Pirson, I., Maenhaut, C., and Christophe, D. (1966) *Endocrinology* **137**, 4691–4698
  45. Rossi, D. L., Acebrón, A., and Santisteban, P. (1995) *J. Biol. Chem.* **270**, 23139–23142
  46. Kambe, F., Nomura, Y., Okamoto, T., and Seo, H. (1996) *Mol. Endocrinol.* **10**, 801–812
  47. Sato, K., and Di Lauro, R. (1996) *Biochem. Biophys. Res. Commun.* **220**, 86–93

**Transcriptional Control of the Forkhead Thyroid Transcription Factor TTF-2 by Thyrotropin, Insulin, and Insulin-like Growth Factor I**

Lourdes Ortiz, Mariastella Zannini, Roberto Di Lauro and Pilar Santisteban

*J. Biol. Chem.* 1997, 272:23334-23339.

doi: 10.1074/jbc.272.37.23334

---

Access the most updated version of this article at <http://www.jbc.org/content/272/37/23334>

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 44 references, 12 of which can be accessed free at <http://www.jbc.org/content/272/37/23334.full.html#ref-list-1>