The EMBO Journal Vol.16 No.11 pp.3185–3197, 1997

TTF-2, a new forkhead protein, shows a temporal expression in the developing thyroid which is consistent with a role in controlling the onset of differentiation

Mariastella Zannini, Virginia Avantaggiato[1], Elio Biffali, Maria Ina Arnone, Koichi Sato, Michele Pischetola, Benjamin A.Taylor[2], Sandra J.Phillips[2], Antonio Simeone[1] and Roberto Di Lauro[3]

Stazione Zoologica 'A.Dohrn', Villa Comunale, 80121 Napoli, Italy, [1]Istituto Internazionale di Genetica e Biofisica, via Marconi 10, Napoli, Italy and [2]The Jackson Laboratory, Bar Harbor, ME, USA

[3]Corresponding author

Expression of thyroglobulin (Tg) and thyroperoxidase (TPO) genes in thyroid follicular cells occurs in the mouse at embryonic day (E)14.5. Two transcription factors, TTF-1 and Pax-8, have been implicated in transcriptional activation of Tg and TPO, even though the onset of their expression is at E9.5, suggesting that additional events are necessary for transcriptional activation of Tg and TPO genes. We report in this paper the cloning of TTF-2, a DNA binding protein that recognizes sites on both Tg and TPO promoters. TTF-2 is a new forkhead domain-containing protein whose expression is restricted to the endodermal lining of the foregut and to the ectoderm that will give rise to the anterior pituitary. TTF-2 shows transient expression in the developing thyroid and anterior pituitary. In the thyroid, TTF-2 expression is down-regulated just before the onset of Tg and TPO gene expression, suggesting that this transcription factor plays the role in development of a negative controller of thyroid-specific gene expression.

Keywords: anterior pituitary/differentiation/thyroid/transcription/TTF-2

Introduction

The genetic mechanisms responsible for differentiation in multicellular organisms ultimately converge upon the transcriptional regulation of genes that are expressed exclusively in a specific cell type. Several transcription factors have been identified that bind to and activate transcription from promoters of cell type-specific genes (Mitchell and Tjian, 1989; Simmons *et al.*, 1990; De Simone and Cortese, 1991; Damante and Di Lauro, 1994). Interestingly, most of the transcription factors thus identified have been detected not only in cells that express the genes that they were originally discovered to activate but also in a few other cell types that display distinct differentiation programs. The emerging picture is that each cell type contains a specific combination of transcription factors and that cooperation among them (Herschlag and Johnson, 1993; Holloway *et al.*, 1995, and references therein) or with non-DNA binding cofactors (Luo *et al.*, 1992; Gstaiger *et al.*, 1995; Strubin *et al.*, 1995) is responsible for the specification of distinct transcriptional phenotypes. Some of the mechanism(s) responsible for cooperation have recently started to be elucidated (Tjian and Maniatis, 1994). Another finding of great interest is that several of the transcription factors originally identified as activators of genes expressed in terminally differentiated cells play a more general role in the survival and/or expansion of the differentiated cell population. In fact, mice homozygous for null alleles of certain cell type-specific transcription factors (Jonsson *et al.*, 1994; Lin *et al.*, 1994; Kimura *et al.*, 1996) show a complete subversion or even absence of the differentiated tissue. Probably related to this observation is the fact that some cell type-specific transcription factors appear in development long before expression of the target genes (Plachov *et al.*, 1990; Lazzaro *et al.*, 1991; Poleev *et al.*, 1992; Lamonerie *et al.*, 1996), suggesting that they may play an additional role(s) during organogenesis.

We have been using specific expression of the thyroglobulin (Tg) and thyroperoxidase (TPO) genes in follicular cells of the thyroid gland as an experimental system to investigate the genetic mechanisms responsible for differentiation in vertebrates. By DNA binding assay, carried out on the functionally relevant regions of Tg and TPO promoters, three factors were identified: TTF-1, TTF-2 and Pax-8. The cloning of TTF-1 (Guazzi *et al.*, 1990) and Pax-8 (Plachov *et al.*, 1990) allowed the demonstration of their nature as 'bona fide' transcriptional activators, since expression of either factor in non-thyroid cells induced transcription from co-transfected Tg and TPO promoters, albeit with a strong preference of TTF-1 for the former and Pax-8 for the latter. Studies on the timing of TTF-1 (Lazzaro *et al.*, 1991; Kimura *et al.*, 1996; this study) and Pax-8 (Plachov *et al.*, 1990; Zannini *et al.*, 1994) expression during development of thyroid follicular cells in the rat and in mouse provided evidence that both TTF-1 and Pax-8 are present in the thyroid primordium at embryonic day (E)9.5, while expression of the known target genes Tg and TPO begins only at around E14. These observations suggest that between E13 and E15 important events must occur that trigger the action of TTF-1 and Pax-8 on the promoters of Tg, TPO and, presumably, other thyroid-specific genes.

In this paper we describe the cloning of TTF-2 cDNA from a rat thyroid cDNA library. To date, TTF-2 has been identified as a thyroid-specific DNA binding factor whose activity is sensitive to insulin, IGF-1 and thyroid stimulating hormone (Santisteban *et al.*, 1992; Aza-Blanc *et al.*, 1993). The functional relevance of the interaction between TTF-2 and the Tg or the TPO promoters has been implied by the effect of mutations in the TTF-2 binding site of each promoter (Sinclair *et al.*, 1990; Francis-Lang *et al.*, 1992). We show in this paper that TTF-2 is a new member

of the family of proteins characterized by the presence of a forkhead domain. TTF-2 mRNA shows a very restricted tissue distribution, being transiently expressed only in the thyroid and in the embryonal anterior pituitary. In thyroid, TTF-2 mRNA, first detected at E9, disappears between E13 and E15. The precise correlation between TTF-2 down-regulation and the triggering of thyroid differentiation suggests that TTF-2 might function as a negative regulator of differentiation in the early stages of thyroid morphogenesis. In keeping with such a model, we demonstrate that TTF-2 represses transcriptional activation by both TTF-1 and Pax-8.

Results

Cloning of a cDNA encoding a new forkhead domain-containing protein

The DNA sequence recognized by TTF-2 is similar to that recognized by transcription factors containing a forkhead domain (Sato and Di Lauro, 1996). Hence, a rat thyroid library was screened with a DNA fragment derived from plasmid pCMV3β (kindly provided by Dr R.H.Costa) corresponding to the region encoding the forkhead domain of the transcription factor HNF3β. Partial sequencing of the 12 positive clones isolated revealed that they all encoded the same novel protein (data not shown). One of the clones (Figure 1A, cl13), containing an insert ~2500 bp long, included all the others and was completely sequenced, revealing an open reading frame (ORF) 1347 nt long that was followed by a 3′-untranslated region (UTR) of 1100 bp. Since the ORF began at one end of the clone it was important to verify whether cl13 contained the entire coding information of the corresponding gene. A restriction fragment from the 5′-end of cl13 was used as a probe to screen a rat genomic library and to isolate a genomic clone (data not shown), from which a 1.9 kb fragment, positive to the screening probe was subcloned in Bluescript (Figure 1A, cl3.2) and completely sequenced. The nucleotide sequence of clone cl3.2 overlapped extensively with cl13 and extended for 970 bp upstream. RNase mapping experiments were then performed to determine the extent of transcribed sequences present in cl3.2 (Figure 1B). The three riboprobes used had a common 5′-end, located in a region overlapping with cDNA clone cl13, and extended for various lengths into the genomic clone. Riboprobe 1 was completely protected by thyroid RNA, while riboprobes 2 and 3 were partially protected, yielding fragments of identical size. This experiment demonstrates that the mRNA from which cl13 is derived must extend into the sequence of clone cl3.2, in a region between the 3′-ends of riboprobes 2 and 3 (Figure 1B), whose precise location could be deduced by the size of the protected fragments. This information was used to build the structure of a putative mRNA obtained by fusing the sequence of clone cl13 and 355 nt from cl3.2. These conclusions were supported by RT-PCR experiments showing that when thyroid cDNA was used as a template it was possible to amplify the sequences downstream of the putative transcription start site identified by RNase mapping, while there was no amplification if one of the primers was derived from the sequence upstream of it (data not shown). The composite sequence of Figure 2A has also been confirmed by sequencing of genomic clones, which further

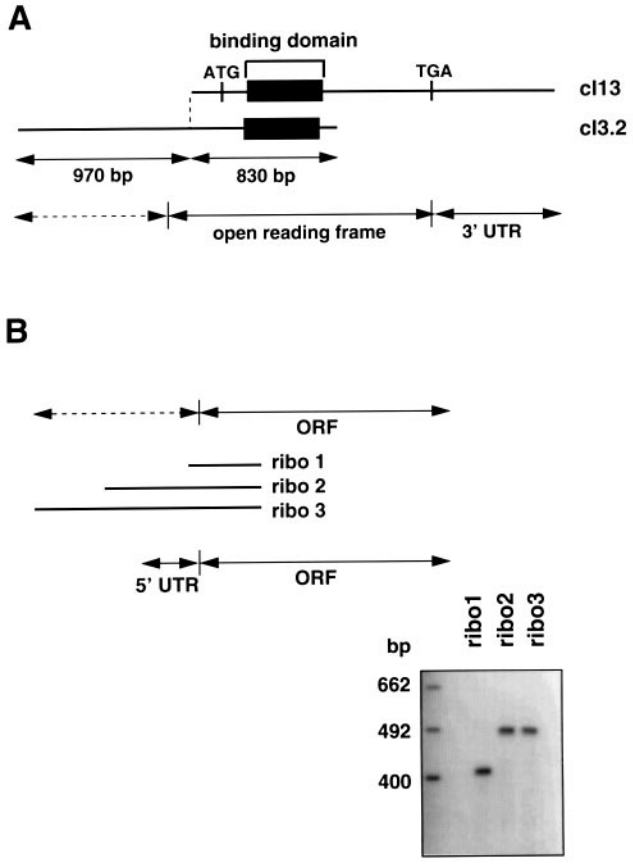

Fig. 1. Structure of the TTF-2 clones and determination of the transcription start site. (**A**) Schematic representation of the longest positive cDNA clone (cl13) and of the partially overlapping genomic subclone (cl3.2). The ORF and the 3′-UTR are indicated. (**B**) RNase mapping experiments were carried out to determine the 5′-UTR of the cDNA with three different riboprobes (ribo 1, ribo 2 and ribo 3). Riboprobes 2 and 3 protect a fragment of identical size (~490 bp), longer than that protected by riboprobe 1. The resulting 5′-UTR is indicated in the scheme.

revealed the absence of introns in the corresponding genomic region (data not shown).

The cDNA sequence in Figure 2A shows a long 5′-UTR, containing an ATG codon at position 212, surrounded by a good Kozak consensus sequence and followed by an in-frame stop codon at position 248. After the stop codon, a long ORF begins which encodes, between its first ATG codon at position 593 and the stop codon at nt 1702, a 370 amino acid protein with a predicted relative molecular mass of 42 kDa. To establish the size of the protein encoded, the sequence contained between nt 1 and 1705 of Figure 1 was amplified from a full-size genomic clone and subcloned in Bluescript (construct IVT 1 of Figure 2B). Three mutants of IVT 1 were constructed (Figure 2B): IVT 2 is identical to IVT 1 except that the first ATG is mutated to GTG; in IVT 3 the first ATG is deleted, together with all the sequences upstream of the second ATG; this ATG is mutated to GTG in IVT 4, which is otherwise identical to IVT 3. mRNAs were transcribed from all four constructs, translated in an *in vitro* reticulocyte lysate system in the presence of [^{35}S]methionine and the resulting products visualized by autoradiography (Figure 2B). mRNAs obtained from IVT 1, 2 and 3 all directed synthesis of a protein of 42 kDa

A

```
   1 ctggcgcctttaaggaggcgaagccagcggagggaggagctggcccaggtgtgtgcaggagagcgcctcgccggcgggac  80
  81 tcggggctggagtccagggcggcagagcggagcagttgctcgccacccggcccggcccactcgcccgcctcgccactggc 160
 161 ttcgcctctctgccctccgcttgggctgtatcttcaccgcagaggtgcggc ATG GGC GGC CAC CGC AGG GGT 232
                                                        M   G   G   H   R   R   G

 233 GTA CGT TTC CCC CAC TGA  CTG CTC CTG CAC GAG ACC CCA GCC CGC AGC CTC CTC TAC GCG 292
      V   R   F   P   H   *    L   L   L   H   E   T   P   A   R   S   L   L   Y   A

 293 CAC CGC CTC GAC AGT CAT CCT CCG ACC CGC AGG AAC TCC CAA ACA GTT GCC ACC ACC CGA 352
      H   R   L   D   S   H   P   P   T   R   R   N   S   Q   T   V   A   T   T   R

 353 GCC TCC GAG CCA CCT CTG GAC CCC CTG ATC TCT TGG CAC CAG AAC TTC AAG GAC CCT CTG 412
      A   S   E   P   P   L   D   P   L   I   S   W   H   Q   N   F   K   D   P   L

 413 GCC CGT GCC CCG CAG GAG CCG TCG GTG GAC GCC GCG CTC CAG ACC CCG AAG TCC CCT GCT 472
      A   R   A   P   Q   E   P   S   V   D   A   A   L   Q   T   P   K   S   P   A

 473 CTC AGT GCT CTC CGT GCC AGC CGC GAA GCC CGG CAG AGT CGG CTC TCG GGG TTC GGG ATC 532
      L   S   A   L   R   A   S   R   E   A   R   Q   S   R   L   S   G   F   G   I

 533 TGG GGC CGG CGC GGC CGT CCG CAG CTG CAG ACG ACG GGG GGT ACG GGG GAC CGC GGC GCC 592
      W   G   R   R   G   R   P   Q   L   Q   T   T   G   G   T   G   D   R   G   A

 593 ATG ACA GCC GAG AGC GCG CCG CCC CCG CCG CAG CCC GAG GCG CTG GCG GCC GTG AAG 652
      M   T   A   E   S   A   P   P   P   P   Q   P   E   A   L   A   A   V   K

 653 GAA GAG CGC GGT GAG GCG GCG GCG GGC GCG GGG GTC CCG GCG GAG GTG GCG GGT CGC GGC 712
      E   E   R   G   E   A   A   A   G   A   G   V   P   A   E   V   A   G   R   G

 713 GCC GGA GGA CGG CGA CGC AAG CGC CCC CTG CAG CGG GGG AAG CCG CCG TAC AGC TAC ATC 772
      A   G   G   R   R   R   K   R   P   L   Q   R   G   K   P   P   Y   S   Y   I

 773 GCA CTC ATC GCG ATG GCT ATC GCG CAC GCG CCC GAG CGC CGC CTG ACT CTG GGC GGC ATC 832
      A   L   I   A   M   A   I   A   H   A   P   E   R   R   L   T   L   G   G   I

 833 TAC AAG TTC ATC ACC GAG CGC TTC CCG TTC TAC CGC GAC AAC CCC AAG AAG TGG CAG AAC 892
      Y   K   F   I   T   E   R   F   P   F   Y   R   D   N   P   K   K   W   Q   N

 893 AGC ATC CGC CAC AAC CTC ACC CTC AAC GAC TGC TTC CTC AAG ATC CCG CGC GAG GCG GGC 952
      S   I   R   H   N   L   T   L   N   D   C   F   L   K   I   P   R   E   A   G

 953 CGC CCG GGC AAA GGC AAC TAC TGG GCG CTC GAC CCC AAC GCC GAG GAC ATG TTC GAG AGC 1012
      R   P   G   K   G   N   Y   W   A   L   D   P   N   A   E   D   M   F   E   S

1013 GGC AGC TTC CTG CGC CGG CGC AAG CGC TTC AAG CGC TCG GAC CTG TCC ACC TAC CCG GCG 1072
      G   S   F   L   R   R   R   K   R   F   K   R   S   D   L   S   T   Y   P   A

1073 TAC ATG CAT GAT GCA GCC GCT GCT GCC GCC GCC GCC GCC GCC GCC ATC TTC CCG GGT GCC 1132
      Y   M   H   D   A   A   A   A   A   A   A   A   A   A   I   F   P   G   A

1133 GTG CCT GCC GCG AGG CCG GCC TAC CCG GGA GCC GTC TAT GCG GGC TAC GCG CCG CCG CTC 1192
      V   P   A   A   R   P   A   Y   P   G   A   V   Y   A   G   Y   A   P   P   L

1193 GCC GCA CCC CCG CCG GTC TAC TAC CCC GCA GCG TCG CCC GGG CCC TGC CGG GTC TTC GGC 1252
      A   A   P   P   P   V   Y   Y   P   A   A   S   P   G   P   C   R   V   F   G

1253 TTG GTT CCC GAG CGG CCG CTC AGC CCG GAT CTG GGC CCC GCA CCA TCA GCA GCC GGA GGT 1312
      L   V   P   E   R   P   L   S   P   D   L   G   P   A   P   S   A   A   G   G

1313 TCG TGC GCC TTC GCG GCT GCG GCG GGC GCG CCG GGC ACA GGG AGC TTC CAG CCC GCG GTA 1372
      S   C   A   F   A   A   A   A   G   A   P   G   T   G   S   F   Q   P   A   V

1373 TGC ACG GGC GCG CGG CCG GTC AAC CCG GCT GCC TAT GCC GCT GCC TAC GCG GGC CCC GAC 1432
      C   T   G   A   R   P   V   N   P   A   A   Y   A   A   A   Y   A   G   P   D

1433 GGC GCG TAC CCG CAA GGG GCG AGC AGC GCG CTC TTC GCC GCT GCT GCG GGC CGT CTG GCC 1492
      G   A   Y   P   Q   G   A   S   S   A   L   F   A   A   A   A   G   R   L   A

1493 GGA CCC ACT TCG CCC ACC GCG GGA GGA GGC AGC GGC GGC GTG GAG GCC ACG GTG GAC TTC 1552
      G   P   T   S   P   T   A   G   G   G   S   G   G   V   E   A   T   V   D   F

1553 TAC GGG CGC ACG TCT CCT GGC CAG TTC GGA GCG GCG CTG GGG CCC TGC TAT AAT CCC AGT 1612
      Y   G   R   T   S   P   G   Q   F   G   A   A   L   G   P   C   Y   N   P   S

1613 GGG CAA CTC GGA GCC GGT GGT GGA GGT GCC TAT CAC TCC CGC CAC GCC ACC GCC TAC CCG 1672
      G   Q   L   G   A   G   G   G   G   A   Y   H   S   R   H   A   T   A   Y   P

1673 GGC GCG GTG GAT CGG TTC GTG TCT GCC ATG TGA gccgggggtaggcacaaaacctcactgactccggca 1741
      G   A   V   D   R   F   V   S   A   M   *

1742 cgttctgaaagatgtaaatccccccggaaccggtggacaggactaaaagagtatcggactgagactcacgagtgtgcgagg 1821
1822 gctccgagggtcgcggaggctcggtgtcttgccctgttcacagaccggatttgctttggacgtgggcggacgtagggagg 1901
1902 ccatagccacgtttgggctgacacaggggaccctcgatgatgcgaagctccaaagagcatgcctttccgccattctacta 1981
1982 gggagggccctagctgtaaagggtggagtgttgcgttgttgcccgcaccagaatctagaatcctgaactgtggtggagtc 2061
2062 gtttggcttcagcgtgtgctatgctgggctaaactcgggctttttacacgttcgcctcttaacttttgactaccgtaccc 2141
2142 caaataaaaggctcggacagtgcctttataattaaagtgagagcaagtttgcaaaaagagttgctttttaaaaattagtt 2221
2222 cggttcagctgggttagttttccctgcctctgagagtcacaagcctgggtcgcaatcgtaactgaattctcggtgtgatc 2301
2302 cagccctaacacctgaattttgagtctgtctgttatacagaactgtgcgattccgccgtctcatttttaaaccccctccc 2381
2382 attatcttgggtgccaaccttccatatttggcagcattggtcaaccacctagggcttcccagtagccatgacagaaggcc 2461
2462 atccaggaatgggaacagaacattggcaagtatttaacccaaagttttaggactggctccaagtagagattgtgcttaat 2541
2542 tattgatttttttttttgttgttgttgttttgagagagagagagagattggtcaggccgcagcagtgactacccagtca 2621
2622 aaaatctgattttctgataagaatgtaggtggggagactttccttaactggggcttgtctgtttctgtttccctgtgcgctc 2701
2702 cttcttcggcgccctcctcgaacaaaaaggaaaagtaataaattagatggttaacaaagagatctttttttaatttttta 2781
2782 tttcattctgtatcttgtttgttttg                                                        2807
```

B

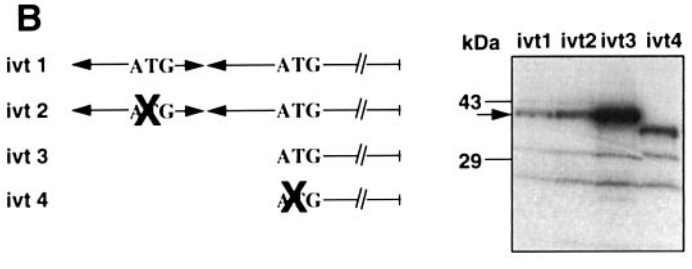

Fig. 2. Sequence of the TTF-2 cDNA and structure of the encoded protein. (**A**) Nucleotide sequence of rat TTF-2 cDNA and deduced amino acid sequence. The conserved forkhead motif is boxed. The two poly(A)$^+$ addition sites identified in the 3′-UTR are underlined. (**B**) Schematic representation of wild-type (ivt1) and mutated (ivt2, ivt3 and ivt4) TTF-2. The different constructs were *in vitro* translated and the reactions were analyzed by 10% SDS–PAGE. Protein molecular weight markers are indicated. The arrow indicates the size of wild-type TTF-2 protein.

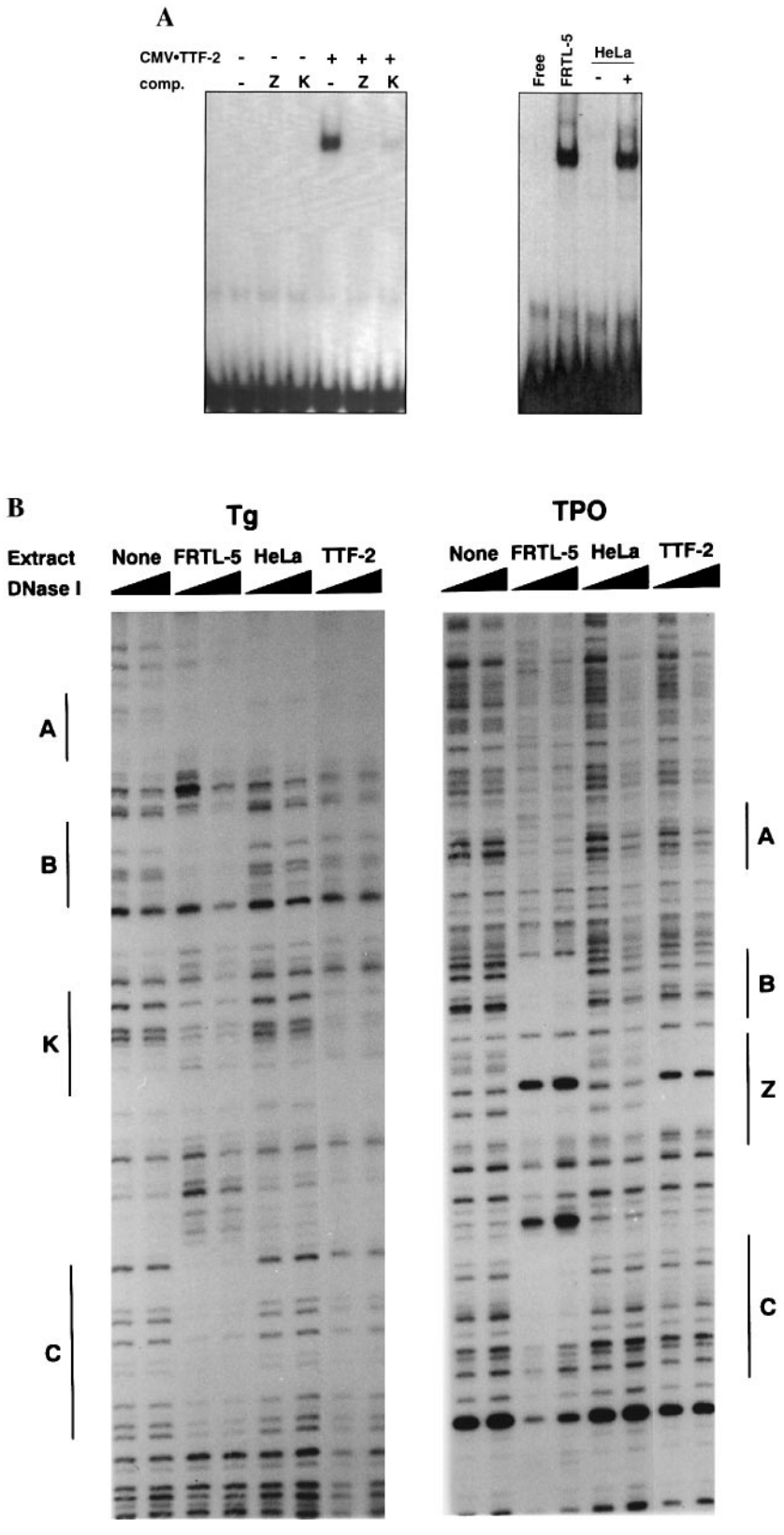

Fig. 3. DNA binding properties of the cloned TTF-2. (**A**) (Left) Gel mobility shift assay using as a probe the K oligonucleotide derived from the Tg promoter (Sinclair *et al.*, 1990). The labeled oligonucleotide was incubated in the presence of total extracts from HeLa cells and HeLa cells transiently transfected with an expression vector for TTF-2. A fixed amount (100×) of unlabeled competitor oligonucleotide K or Z (Francis-Lang *et al.*, 1992) was used, as indicated above the lanes. (Right) To demonstrate co-migration, a band shift experiment with the same oligonucleotide was carried out with extracts from FRTL-5 and mock and CMV·TTF-2-transfected HeLa cells. (**B**) Comparison of DNase I footprinting obtained on the thyroglobulin (Tg) and thyroperoxidase (TPO) promoters with total extracts of FRTL-5 cells, HeLa cells and HeLa cells expressing exogenous TTF-2. All the footprints obtained with FRTL-5 extracts are shown. The region protected by extracts of HeLa cells expressing TTF-2 corresponds to the K and Z footprints in the Tg and TPO promoters respectively.

relative molecular mass, which is consistent with initiation at the ATG in position 593. This indication was confirmed by the specific sensitivity of only the second ATG to mutagenesis, as indicated by the disappearance of the 42 kDa protein with the mRNA transcribed from the IVT 4 template. In this case, a new, smaller protein is obtained, probably initiating at a spurious downstream initiation site.

The most notable feature of the predicted protein is a forkhead domain, encoded by nt 746–1045 (boxed in Figure 2A). Two polyadenylation sites are present in the 3′-UTR (underlined in Figure 2A) which are alternatively used to produce two differently sized mRNAs (Figure 4).

The cloned cDNA encodes a protein that displays the same binding properties as TTF-2

The full-length cDNA was subcloned in an expression vector under the control of a CMV promoter and the construct thus generated (CMV·TTF-2) was transiently transfected into HeLa cells. Extracts prepared from transfected cells contained a DNA binding activity capable, in a band shift assay, of forming a complex with oligonucleotide K (Figure 3A, left panel), derived from the TTF-2 binding site in the Tg promoter (Sinclair *et al.*, 1990). The complex was competed for by a molar excess of unlabeled oligonucleotides containing the TTF-2 binding site from the Tg (K) or TPO (Z) promoters (Francis-Lang *et al.*, 1992) and it was not observed with extracts prepared from mock-transfected HeLa cells (Figure 3A, left panel). Furthermore, a complex of similar mobility was obtained with extracts derived from the thyroid cell line FRTL-5 (Figure 3A, right panel).

To further investigate the binding properties of the protein encoded by the cDNA that we have cloned, a DNase I footprinting analysis was performed on the Tg and TPO promoters. As shown in Figure 3B, footprints obtained with an FRTL-5 nuclear extract were compared with that obtained with extracts of HeLa cells transfected with either an empty expression vector (HeLa lanes in Figure 3B) or with CMV·TTF-2 (TTF-2 lanes in Figure 3B). In the Tg promoter the region specifically protected by the protein expressed in transfected HeLa cells coincided with the footprint obtained with the FRTL-5 extract previously described as being TTF-2 (region K, Figure 3B). The same results were obtained in the TPO promoter (region Z, Figure 3B).

These data strongly indicate that the cDNA cloned has the same binding properties as previously described for TTF-2.

Chromosomal localization of the TTF-2 gene

High stringency hybridization of the labeled TTF-2 clone to Southern blots of C57BL/6J and SPRET/Ei DNA digested with *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, *Nsi*I, *Sst*I and *Msp*I revealed a distinct variant only with *Sst*I, suggesting that this variant reflects a small DNA sequence change rather than a large insertion/deletion mutation (data not shown). The C57BL/6J and SPRET/Ei *Sst*I fragments were 4.3 and 5.1 kb respectively. Ninety four BSS interspecific backcross progeny (Rowe *et al.*, 1994) were scored with respect to the presence or absence of the 4.3 kb TTF-2 *Sst*I fragment from C57BL/6J. The locus thus identified is designated *titf2*. No recombinants

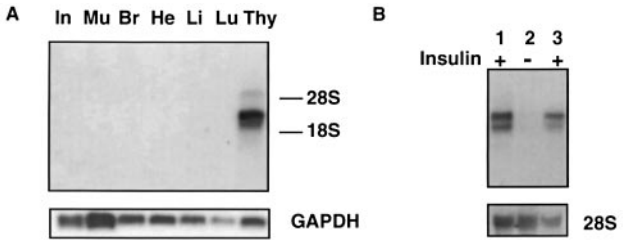

Fig. 4. Tissue-specific expression and insulin regulation of TTF-2 mRNA. (**A**) Northern blot analysis of TTF-2 transcripts in rat adult tissues. Poly(A)$^+$ RNAs (2 μg) from several adult mouse tissues were hybridized with a TTF-2 specific probe. Two transcript of 2.8 and 2.3 kb were detected only in thyroid. In, intestine; Mu, muscle; Br, brain; He, heart; Li, liver; Lu, lung; Thy, thyroid. GADPH hybridization was used to normalize the amounts of RNA present in the blots. (**B**) Northern blot analysis of total RNA from FRTL-5 cells. Total RNA (30 μg) from FRTL-5 cells grown in the presence of insulin (lane 1), after 4 days insulin starvation (lane 2) and after re-addition of insulin to cells that had been previously starved (lane 3) were hybridized to the same probe as in (A). 28S hybridization was used to normalize the amounts of RNA present in the blots.

(0/93) were found between *titf2* and the previously scored *Xpa* (xeroderma pigmentosum complementation group A) locus on chromosome 4 (Chidambaram and Dean, 1996). The 95% upper confidence limit of the *titf2–Xpa* map distance is 3.2 cM. *titf2* mapped 9.6 ± 3.0 cM distal to *D4Mit4* and 8.5 cM proximal to *Orm1* (orosomucoid-1). These loci have been placed at positions 10.5 and 30.6 cM from the centromere in the mouse chromosome 4 map (Mock *et al.*, 1996). The present data suggest that *titf2* is located ~22 cM from the centromere. The *Xpa* locus has been assigned to cytogenetic band 4C2 (Tanaka *et al.*, 1990), while the human homolog of *Xpa* maps to 9q22.3, suggesting that *titf2* and its human homolog will also map to the same regions. No mouse mutations affecting thyroid function have been mapped to the region of chromosome 4 containing *titf2*.

TTF-2 is expressed in adult thyroid and shows a tight hormonal control in cultured thyroid cells

To examine the tissue distribution of TTF-2 mRNA, total RNA was prepared from various adult rat tissues and analyzed by Northern blot hybridization using a 0.35 kb probe derived from the 3′ non-coding region. TTF-2 showed a remarkable tissue-specific expression (Figure 4A), being present, among the tissues examined, only in the thyroid. Two mRNAs, of 2.8 and 2.3 kb, were observed; Northern blots with specific oligonucleotides and sequencing of the 3′-UTR of several cDNA clones (data not shown) showed that the two transcripts originate by alternative use of the two polyadenylation signals identified in the 3′-UTR of the longest cDNA (see Figure 2A).

TTF-2 binding activity is under strict insulin/IGF-1 control, as it becomes undetectable in nuclear extracts from cells starved for insulin and can be induced upon re-addition of insulin/IGF-1 in a dose-dependent manner (Santisteban *et al.*, 1992). We thus chose regulation by insulin as an additional criterion to demonstrate that the cDNA that we isolated was indeed TTF-2. FRTL-5 cells were grown in medium depleted of insulin for 4 days and total RNA was prepared. Northern blot analysis showed that expression of our cDNA clone was strictly regulated by insulin, being detectable only in cells grown in regular

medium or upon re-addition of insulin to cells that had been previously starved (Figure 4B).

TTF-2 expression during embryonic development

A 350 bp fragment derived from the 3′-UTR was used as the probe to study TTF-2 expression during mouse development between E7.5 and E17, performing *in situ* hybridizations on embryo sections. Control experiments using as a probe the sense strand of the same fragment showed no detectable signals (data not shown). The first detectable signal was found at E8.5 in the endoderm corresponding to the floor of the foregut (Figure 5A). At E9.2–9.5 TTF-2 was expressed in the epithelium lining the anterior foregut and the posterior stomodeum, including the pharyngeal membrane (Figure 5B). At E10.5 TTF-2 was transcribed along all the endoderm of the foregut lining the visceral pouch of the branchial arches (Figure 5C, D and F). Rostrally in the stomodeum TTF-2 expression was confined to the dorsal ectoderm (Figure 5C–E and G). However, TTF-2 mRNA appeared undetectable in the most rostral ectoderm which lines the nasal process and the olfactory placode (Figure 5C, D and G). TTF-2 expression was evident in two endocrine gland primordia: Rathke's pouch, of ectodermal origin, that will form the anterior pituitary, and the thyroid anlage, a small group of endodermal cells that will form the thyroid follicular cell population (Figure 5D and E).

A more detailed analysis of TTF-2 expression in thyroid precursors was performed, comparing it with expression of TTF-1, which is known to begin in thyroid cell precursors at E8.5. At E8.5 TTF-1 was expressed in a small group of cells in the deep midline of the floor of the foregut (Figure 6A). At that time TTF-2 was expressed through the foregut endoderm (see above) even though its expression did not peak in the thyroid early primordium (Figure 6F), but appeared much more evidently in posterior sections (Figure 6G) where TTF-1 was not transcribed (Figure 6B). At E9.5, both TTF-1 and TTF-2 transcripts were detectable in the migrating thyroid primordium (compare Figure 6C with H) and during the following 3 days (E10.5 and E12.5 in Figure 6) expression in the migrating thyroid cell precursors was very clearly detected (Figure 6D, E, I and J). Hence, TTF-1 and TTF-2 appear to be co-expressed in the same cells in a defined temporal window during early thyroid development.

Expression of TTF-2 in the developing pituitary was already evident in those ectodermal cells fated to develop into adenohypophysis that are located in close proximity to the pharyngeal membrane in the inner stomodeum (Figure 7F and F′). It is likely that these cells, which are in direct apposition to the diencephalic floor, play an important role in inducing the neurohypophysis, which has been highlighted by hybridization of adjacent sections with TTF-1, a marker of developing neurohypophysis (Figure 7A–E). Later, at E10.5–11.5, TTF-2 expression accompanied the migrating Rathke's pouch (Figure 7G–I), which moves upward to meet the descending infundibulum (Figure 7B–D). By E12–12.5 Rathke's pouch was not connected with the oral cavity and showed a remarkable proliferative activity. At this stage TTF-2 mRNA was undetectable in the adenohypophysis precursor cells (Figure 7J), while there were still high levels of TTF-1 mRNA in the infundibulum (Figure 7E).

TTF-2 expression inversely correlates with differentiation of thyroid follicular cells

A critical event must occur in thyroid differentiation around E13 and E14, since most of the differentiation program is only activated at E14, even though the known regulators of thyroid-specific gene expression, including, as demonstrated by this study, TTF-2, are already expressed at E13 (Damante and Di Lauro, 1994). Interestingly, at E15, when thyroid cells undergo differentiation, as shown by expression of thyroglobulin (Figure 8F), TTF-2 transcripts became undetectable (Figure 8D), while TTF-1 expression remained high. These data suggest that TTF-2 could function as an inhibitor of differentiation in the early stages of thyroid development, perhaps by interfering with transcriptional activation of thyroid-specific gene expression by TTF-1 and, possibly, Pax-8.

TTF-2 interferes with transcriptional activation by TTF-1 and Pax-8

To test whether TTF-2 is indeed capable of interfering with transcriptional activation mediated by either TTF-1 or Pax-8 we used a co-transfection assay in HeLa cells. We have previously shown that in these cells the Tg promoter is best activated by TTF-1, while Pax-8 preferentially activates TPO transcription. Hence, the inhibitory effect of TTF-2 was tested on either TTF-1-mediated Tg transcription or on Pax-8 activation of TPO. We also tested whether TTF-2 was able to repress TTF-1-dependent transcription on C5-E1b, an artificial promoter that contains multiple binding sites for TTF-1 in front of the E1b TATA box (De Felice *et al.*, 1995). As shown in Figure 9, both TTF-1- and Pax-8-mediated transcriptional activation, on the Tg or TPO promoters respectively (Figure 9A and C, black bars), were strongly interfered with by TTF-2 (Figure 9A and C, hatched bars). Surprisingly, we observed TTF-2-mediated transcriptional repression also on Tg and TPO promoter mutants (Figure 9B and D) in which the TTF-2 binding site was abolished by several base substitutions, suggesting that TTF-2 does not need to bind DNA in order to repress transcription. Nonetheless, two lines of evidence suggest that TTF-2-mediated repression is promoter specific: first, the CMV promoter used in our transfections as an internal control to normalize for transfection efficiency was not affected by TTF-2 (data not shown) and, second, C5E1b, whose transcription is completely dependent on TTF-1, was unaffected by TTF-2 (Figure 9E). To be certain about the promoter selectivity of TTF-2 repression, C5E1b–CAT and Tg–Luc were transfected together, with or without TTF-1 and TTF-2 (Figure 9F). In this experiment Tg and C5E1b were activated by TTF-1 simultaneously, within the same cells; co-expression of TTF-2 only repressed Tg, leaving C5E1b unaffected, thus demonstrating the ability of TTF-2 to discriminate even among promoters activated by the same transcription factor.

Discussion

TTF-2 was identified as a thyroid-specific DNA binding protein able to recognize one binding site on the promoters of thyroglobulin (Civitareale *et al.*, 1989) and thyroperoxidase (Francis-Lang *et al.*, 1992), two genes exclusively expressed in the thyroid. The similarity of the

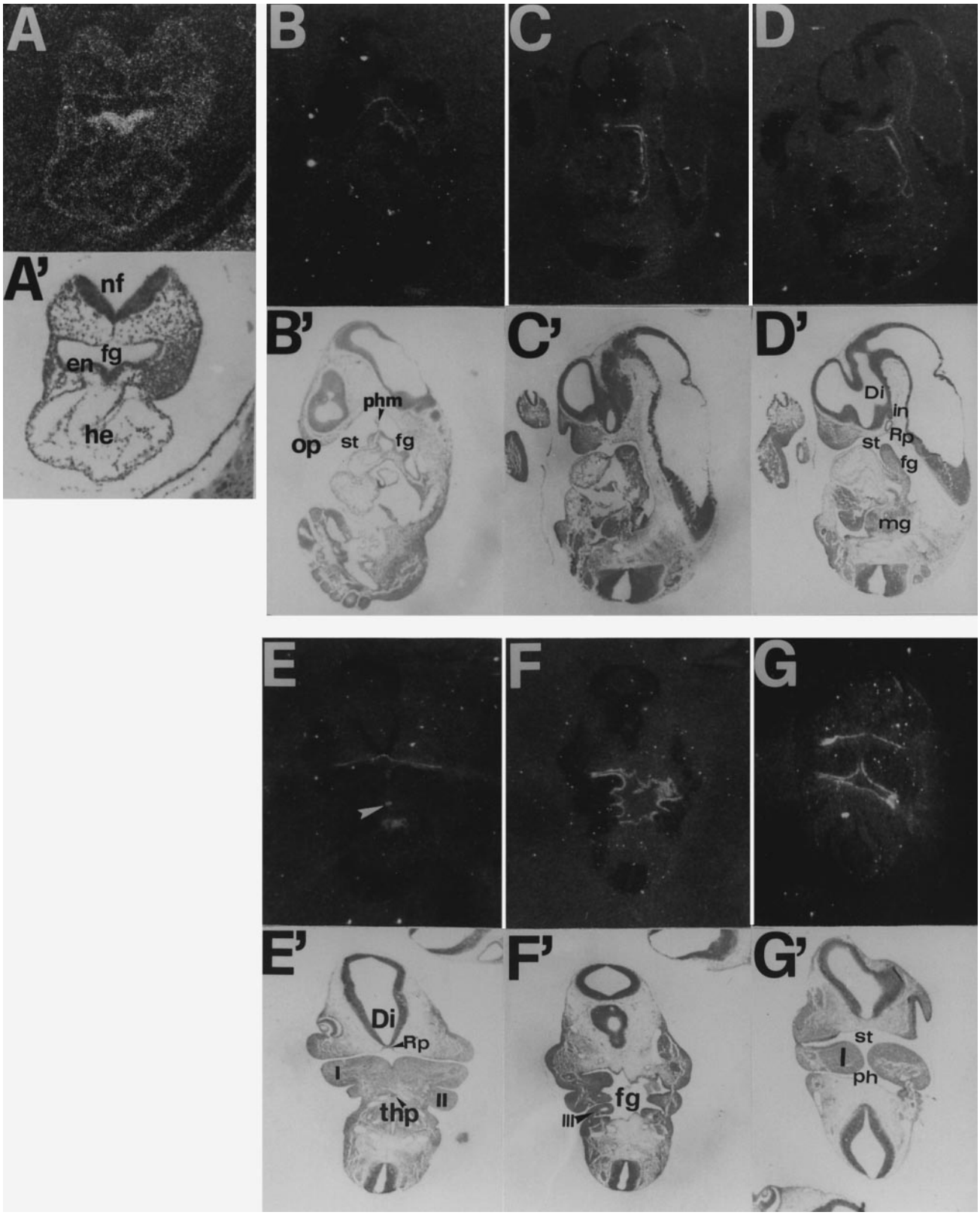

Fig. 5. Expression of TTF-2 during mouse development. TTF-2 expression in transverse (**A** and **G**), sagittal (**B**–**D**) and frontal (**E** and **F**) sections of mouse embryos at E8.5 (A), E9.2 (B) and E10.5 (D–G). Bright fields of the same sections are indicated by a prime. nf, neural fold; fg, foregut; en, endoderm; he, heart; op, olfactory placode; phm, pharyngeal membrane; st, stomatoodeum; Di, diencephalon; in, infundibular diverticulum; Rp, Rathke's pouch; mg, midgut; I, II and III, the first, second and third branchial arches respectively; thp, thyroid primordium; ph, pharynx.

TTF-2 binding site to the consensus sequence recognized by proteins containing a forkhead domain prompted us to isolate cDNA clones encoding forkhead proteins from a thyroid cDNA library. Using this approach, we isolated a cDNA encoding a new forkhead protein that we demonstrate in this study to be TTF-2, on the basis of

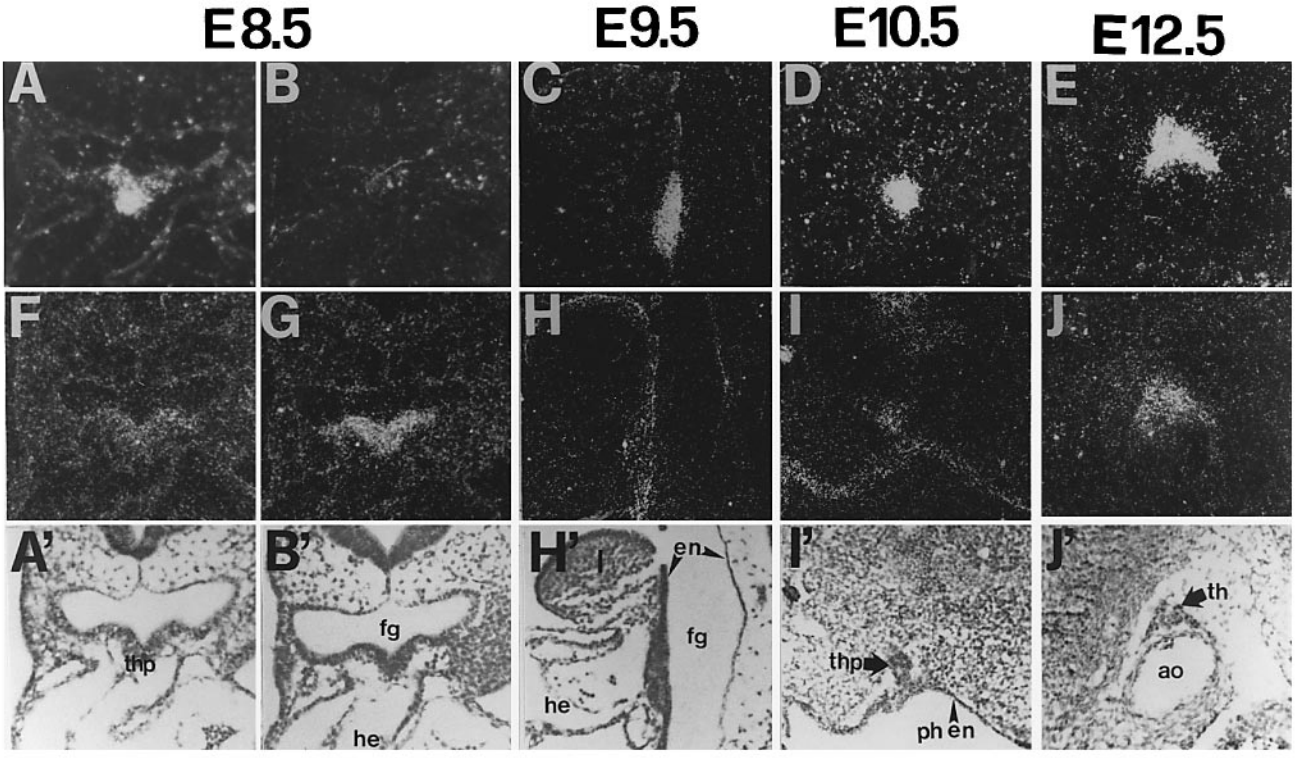

Fig. 6. TTF-2 and TTF-1 expression in the developing thyroid. Detailed comparison of TTF-1 (**A**–**E**) and TTF-2 (**F**–**J**) expression in adjacent sections of thyroid primordium. Embryonic stages are indicated and bright fields of the same sections are indicated by a prime. Abbreviations are as in the previous figures. ao, aorta.

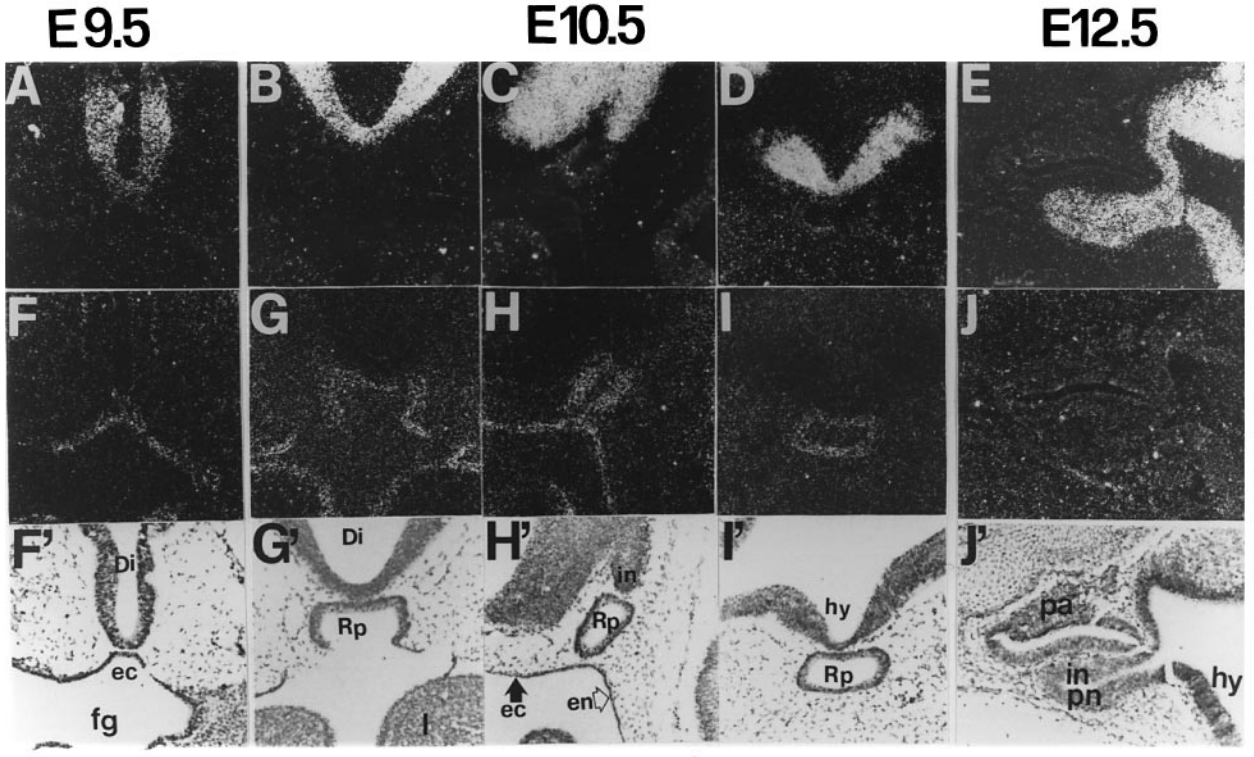

Fig. 7. TTF-2 and TTF-1 expression in pituitary development. Detailed comparison of TTF-1 (**A**–**E**) and TTF-2 (**F**–**J**) expression pattern during pituitary development at E9.5 (A and F), E10.5 (B–D and G–I) and E12.5 (E and J). Bright fields of the same sections are indicated by a prime. (F') and (G'), (H') and (J') and (I') are frontal, sagittal and coronal sections respectively. Abbreviations are as in previous figures. ec, ectoderm; hy, hypothalamus; pn and pa, neural and anterior lobe of the pituitary gland respectively.

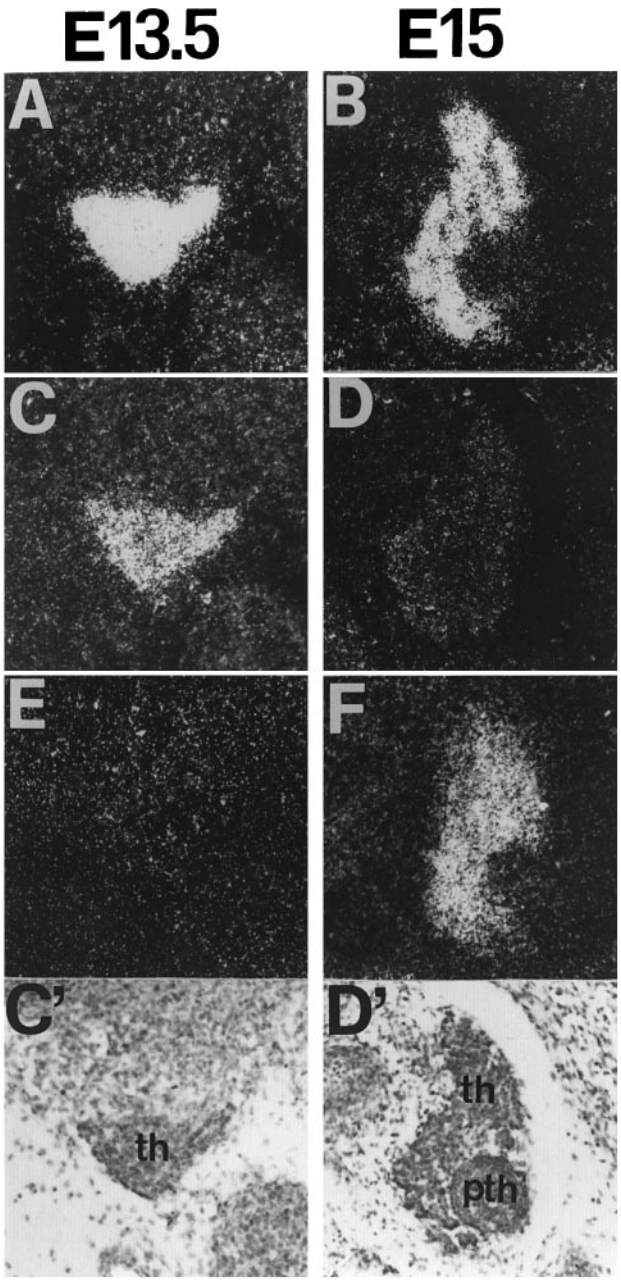

Fig. 8. Appearance of TTF-1 (**A** and **B**), TTF-2 (**C** and **D**) and thyroglobulin (**E** and **F**) mRNA in adjacent sections at E13.5 and E15 of mouse embryo development. Bright fields are indicated with a prime. th, thyroid; pth, parathyroid.

its binding properties, tissue distribution and hormonal regulation.

Of particular interest is expression of TTF-2 during thyroid development. Thyroid follicular cells originate by invagination of pharyngeal endoderm beginning at E8–8.5 of mouse development (Ericson and Frederiksson, 1990). The thyroid primordium migrates downward to reach its final destination in front of the trachea at E13–14. It is only at E15, after completion of the migration process, that thyroid follicular cells differentiate, as measured by expression of several thyroid-specific genes (Tg, TPO and TSHR) (Lazzaro *et al.*, 1991). Two transcription factors, TTF-1 and Pax-8, have been proposed as necessary for thyroid differentiation (Damante and Di Lauro, 1994). The essential role of TTF-1 has been demonstrated by gene targeting experiments, showing that mice homozygous for a null TTF-1 allele fail to develop a thyroid gland (Kimura *et al.*, 1996). Interestingly, both TTF-1 and Pax-8 proteins are present in the thyroid anlage stage at E8.5, suggesting that an additional, essential event(s) must occur to trigger differentiation of thyroid cells at E13–14 (Lazzaro *et al.*, 1991). We show in this paper that TTF-2 expression is turned off exactly between E13 and E15 in the developing thyroid. The correlation between the onset of thyroglobulin and thyroperoxidase gene expression and the disappearance of TTF-2 mRNA suggests that in the embryo the role of TTF-2 is to block activation of thyroid-specific gene expression by TTF-1 and Pax-8. We propose that thyroid cell precursors enter into a determined state at E8.5 which is characterized, and possibly induced, by the presence of TTF-1 and Pax-8. During the next 5 days, thyroid cell precursors undergo a long migration, at the end of which they will express their full differentiated phenotype. The presence of TTF-2 in the migrating thyroid cell precursors would prevent precocious expression of genes that might have an adverse effect on migration, for example because of changes in the adhesive properties of the cells.

In keeping with such a model, we show that TTF-2 is capable of interfering with transcriptional activation by TTF-1 and Pax-8 of Tg and TPO promoters respectively. TTF-2-mediated repression is binding site independent, as demonstrated by the sensitivity to TTF-2 repression of Tg and TPO promoters mutated in the TTF-2 binding sites. Repression of transcription in the absence of a cognate binding site has also been demonstrated for other DNA binding proteins and it is thought to be mediated by protein–protein interactions resulting in interference with assembly of the transcription machinery (Briata *et al.*, 1995; Catron *et al.*, 1995). To exclude a non-specific effect of TTF-2, we determined its effects on other promoters. We observed no repression of the CMV promoter, used in all our transfections as an internal control (data not shown). TTF-2 repression was also tested on C5E1b, an artificial promoter containing five TTF-1 binding sites in front of the E1b TATA box. The C5E1b promoter, similarly to that of Tg, shows a strict dependence on TTF-1 for transcription in HeLa cells. Nevertheless, co-expression of TTF-2 in HeLa cells only repressed Tg and left C5E1b transcription unaffected. Taken together, these data suggest that TTF-2 does not directly interfere with TTF-1 but recognizes either a specific promoter architecture or components of the basal transcriptional apparatus that could be different between Tg and C5E1b, thus also providing a mechanism for interference by TTF-2 with only a subset of the genes activated by TTF-1. This observation is of relevance: the absence of a thyroid in TTF-1 knock-out mice (Kimura *et al.*, 1996) suggests that this factor is involved in morphogenesis and only later on takes up the function of activating transcription of 'terminally differentiated' genes. The discrimination operated by TTF-2 on TTF-1-dependent promoters could be instrumental in repressing differentiation without interfering with morphogenesis.

In adult thyroid tissue TTF-2 expression is restored. Two, albeit indirect, lines of evidence suggest that in the

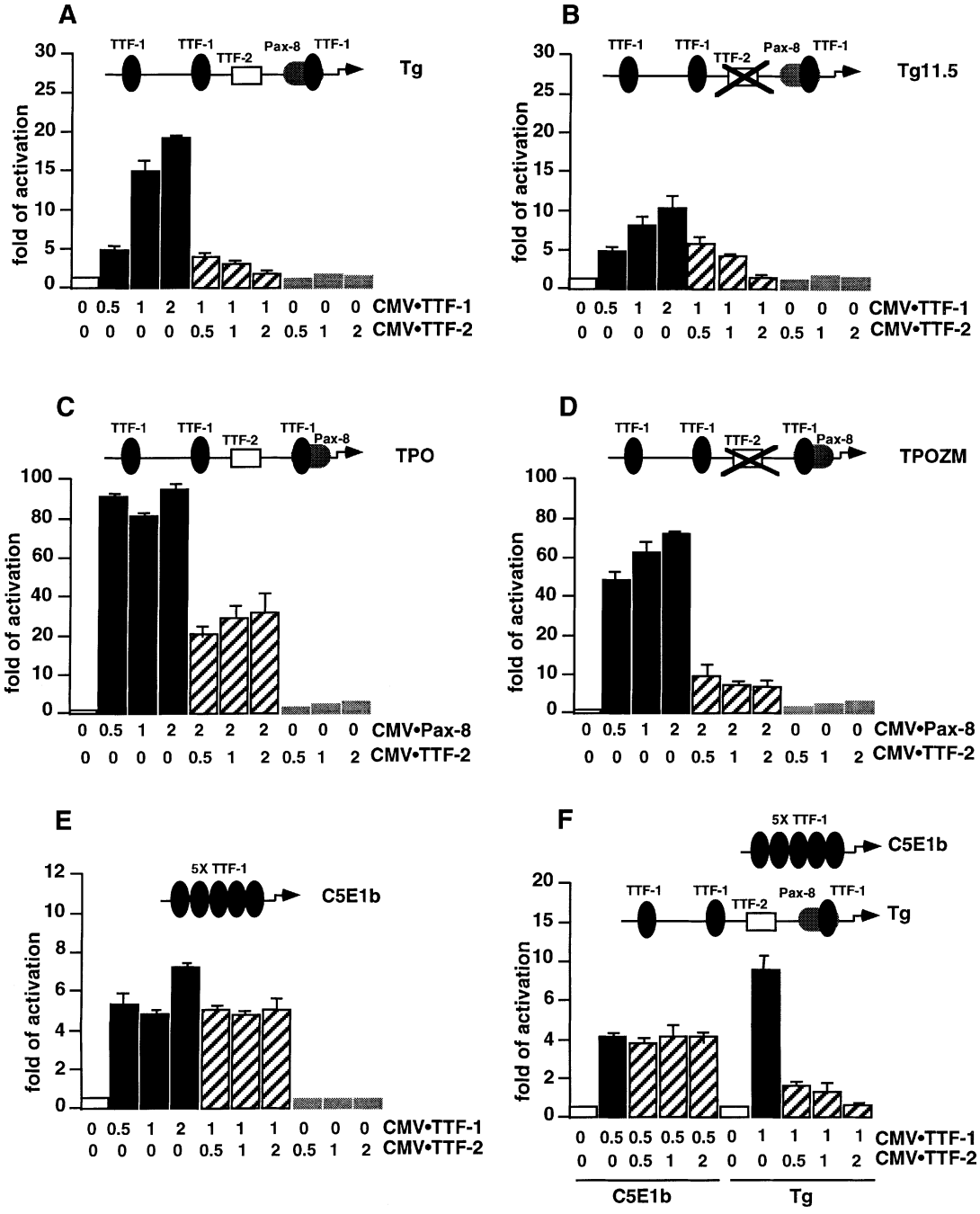

Fig. 9. Interference of TTF-2 with transcriptional activation of the thyroglobulin (Tg) and thyroperoxidase (TPO) promoters by TTF-1 and Pax-8. HeLa cells were transiently transfected with Tg–CAT (**A**), Tg11.5–CAT (**B**), TPO–Luc (**C**), TPOZM–Luc (**D**), C5E1b–CAT (**E**) and Tg–Luc with C5E1b–CAT (**F**) reporter constructs. A schematic structure of each promoter, with the location of the factor binding site, is reported at the top of each diagram. In each panel, the activity of the reporter in the absence of transactivator is indicated by empty bars. Black bars show reporter activity in the presence of either TTF-1 or Pax-8. Hatched bars display the reporter activity when TTF-2 was present together with either TTF-1 or Pax-8, while gray bars indicate activity obtained with TTF-2 alone. Increasing amounts (expressed as μg DNA) of the appropriate expression vector were co-transfected as indicated. The activation values were obtained by dividing the enzymatic activity present in extracts of cells transfected with the various protein encoding expression vectors by the activity obtained with the empty expression vector. The range of activity detected for the reporters was between 0.1 and 2.3% conversion for CAT and between 2000 and 190 000 for luciferase.

adult thyroid TTF-2 functions as a transcriptional activator: first, mutation in the TTF-2 binding site, in both the Tg and TPO promoters, results in reduced transcription (Sinclair *et al.*, 1990; Francis-Lang *et al.*, 1992) and, second, artificial promoters obtained by polymerizing the TTF-2 binding site are transcriptionally active and, furthermore, show hormonal regulation (Aza-Blanc *et al.*, 1993). Even though direct evidence supporting the role of TTF-2 as a transcriptional activator is lacking, it is tempting to speculate that TTF-2 biphasic expression is required to switch from repression to activation of thyroid-specific gene expression. Possibly related to this latter function is binding of TTF-2 to DNA through a highly conserved 110 amino acid domain, the forkhead domain, whose structure, known as the winged helix motif, has also been found in histone H5 (Clark *et al.*, 1993).

The forkhead domain characterizes a large family of transcriptional regulators, also known as *fkh* (Kaestner *et al.*, 1993), *HFH* (Clevidence *et al.*, 1993) or *FREAC* (Pierrou *et al.*, 1994). Similarly to other forkhead domain-containing proteins (Pierrou *et al.*, 1994), TTF-2 is capable of bending DNA (data not shown). Other transcriptional regulators have been shown to exert their transcriptional effects via local modification of DNA bending (Falvo *et al.*, 1995; Giese *et al.*, 1995, and references therein; Thanos and Maniatis, 1995). Such effects could be mediated by an influence on nucleosome positioning in a manner similar to HNF3, another member of the forkhead family that has been demonstrated to be essential for assembly of a multiprotein complex that actively directs nucleosome positioning within the albumin enhancer (McPherson *et al.*, 1993, 1996). In all the cases mentioned, alteration of local DNA or chromatin structure seems to be required for transcriptional activation and could be used by TTF-2 to mediate its putative activating role in adult thyroid cells.

Two other forkhead proteins, HNF3-α and HNF3-β, are expressed in the developing thyroid. Interestingly, both factors show a transient expression pattern, beginning at E10.5 and terminating at E12.5 and E13.5 for HNF3-α and HNF3-β respectively (Monaghan *et al.*, 1993). We have recently shown that, similarly to TTF-2, HNF3-β expression is restored in adult thyroid gland and it is detected in the FRTL-5 cell line (Bohinski *et al.*, 1994). Even though HNF3-β can recognize only the TTF-2 binding site on the TPO promoter and not that in the Tg promoter (Bohinski *et al.*, 1994), its temporal expression pattern in the thyroid is similar to that of TTF-2 and, hence, is consistent with a similar, perhaps redundant, role.

The overall expression pattern of the forkhead proteins HNF3-α, HNF3-β and HNF3-γ in the endoderm of the gastrointestinal tract shows an interesting temporal and spatial, partially overlapping pattern, with HNF3-α and HNF3-β extending most anteriorly in the endoderm lining the oral cavity (Monaghan *et al.*, 1993). HNF3-β expression extends further into ectodermally derived cells in the stomatodaeum and TTF-2 extends even further into Rathke's pouch. Posteriorly, TTF-2 overlaps with the expression domain of HNF3-α and HNF3-β along all the endoderm of the foregut lining the visceral pouch of the branchial arches. In the lung and in all the posterior part of the gut, TTF-2 is absent. Thus it seems that different complements of forkhead proteins and their specific cooperation with other transcription factors could be an important factor in regionalization and differentiation along the foregut axis (Bohinski *et al.*, 1994; Hellqvist *et al.*, 1996).

TTF-2 also shows a transient expression pattern in the developing pituitary. It is first detected in the ectodermal cells fated to develop into adenohypophysis, located in close proximity to the pharyngeal membrane, in direct apposition to those of the diencephalic floor. It is thought that reciprocal inductive interactions occur between the hypothalamic and pituitary primordia and, hence, it is conceivable that TTF-2 gene expression in Rathke's pouch cells is induced by signals originating in the developing neurohypophysis. By E12–12.5, when formation of Rathke's pouch is completed, TTF-2 is undetectable in adenohypophysis precursor cells. The transient expression of TTF-2 in the developing pituitary resembles that of Rpx, a homeodomain-containing protein, whose expression is detected at E7, before the onset of TTF-2 expression. Rpx expression overlaps spatially and temporally with that of TTF-2 and is extinguished 1 day later, at E13.5 (Hermesz *et al.*, 1996). The onset of TTF-2 expression in the developing pituitary just precedes that of α-GSU. Moreover, extinction of TTF-2 expression correlates with the onset of POMC expression, which occurs at E12 in the ventral part of the pituitary primordium (Elkabes *et al.*, 1989). Interestingly, a homeodomain-containing transcription factor, Ptx1, that is capable of activating the POMC promoter begins to be expressed at E10.5 in Rathke's pouch (Lamonerie *et al.*, 1996). By analogy with the model discussed above on the role of TTF-2 in thyroid development, TTF-2 could prevent Ptx1 activation of the POMC promoter. Such a model, which would explain the delay between the onset of Ptx1 expression and activation of POMC transcription, could be tested by assaying the effect of TTF-2 on transactivation of the POMC promoter by Ptx1.

We show in this paper that in the differentiated thyroid cell line FRTL-5 TTF-2 is under tight control by insulin and IGF-1 at the mRNA level. It is worth noting that thyroid-specific expression of TTF-2 coincides in time with embryonic production of IGF-1 (Murphy *et al.*, 1987; Rotwein *et al.*, 1987), suggesting a role for IGF-1 in expression of TTF-2 during development.

TTF-2 is expressed in two glands, the thyroid and the pituitary, which are part of a regulatory circuit responsible for homeostatic control of thyroid hormone production. The hypothalamus is also part of this circuit, where TTF-1, another transcription factor of relevance in thyroid cell function and differentiation, is expressed. In this respect, it is noteworthy that in TTF-1 knock-out mice the anterior pituitary, where TTF-1 is not expressed, is absent, perhaps as a consequence of the absence of hypothalamic structures that are necessary to induce morphogenesis of the adenohypophysis (Kimura *et al.*, 1996). It is inviting to think that recruitment of the same regulatory molecules in different but functionally related organs in the thyroid–pituitary–hypothalamic axis is advantageous to their coordinate development and function.

Materials and methods

Library screening

Samples of 8×10^5 plaques of a rat thyroid cDNA library in λgt11 were plated and duplicate filter lifts were carried out according to standard procedures. Pre-hybridization was carried out at 58°C for 2 h in 6× SSC, 5× Denhardt's, 0.2% SDS, 50 μg/ml salmon sperm DNA, hybridization was performed in the same buffer containing a probe encoding the forkhead domain of HNF3-β labeled by random oligonucleotide priming. Filters were washed twice in 2× SSC, 0.1% SDS. Phage DNA was prepared from purified plaques of the positive clones and the cDNA insert was released by *Eco*RI digestion, subcloned in the Bluescript KS vector and sequenced by the dideoxy method and Thermosequenase (Amersham).

Plasmids

Specific primers were used to amplify the entire coding region or deletion fragments of TTF-2 by PCR. *Eco*RI and *Mlu*I sites (5′-end and 3′-end respectively) were included in these primers to facilitate cloning. The amplified products were subcloned into the eukaryotic expression vector pCMV5. Alternatively, the PCR products were filled in with Klenow and cloned into the *Eco*RV site of the Bluescript KS vector.

Specific methionine codons were changed to alanine codons by PCR as described previously, using primers containing the specific mutations. The mutants thus generated were checked by DNA sequencing.

The plasmids used in transient transfection experiments have been previously described and were as follows: Tg-CAT and Tg11.5-CAT (Sinclair *et al.*, 1990), TPO-LUC and TPOZM-LUC (Francis-Lang *et al.*, 1992), C5E1b-CAT (De Felice *et al.*, 1995), CMV·TTF-1 (De Felice *et al.*, 1995), CMV–Pax-8 (Zannini *et al.*, 1992).

In vitro transcription and translation

For *in vitro* transcription, plasmids IVT 1, IVT 2, IVT 3 and IVT 4, containing the wild-type or mutagenized TTF-2 coding region subcloned in Bluescript, were linearized with a suitable restriction enzyme at the 3′-end of the Bluescript polylinker. The sense RNA strand was synthesized with T3 or T7 RNA polymerase (Promega), according to the cloning orientation. One tenth of the RNA template was translated in a rabbit reticulocyte lysate following the manufacturer's specifications (Promega) and an aliquot was subjected to SDS–PAGE to check the presence and the amount of the protein.

Cell culture and transfection

Cell line FRTL-5 has been previously described in detail (Ambesi-Impiombato and Coon, 1979). Briefly, FRTL-5 cells were grown in Coon's modified F12 medium (Seromed) supplemented with 5% calf serum (Gibco) and six growth factors as described by Ambesi-Impiombato and Coon (1979). HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf medium (Gibco). For transient expression assays, cells were plated at 8×10^5 per 100 mm diameter tissue culture dish 1 day prior to transfection. Transfections were carried out by the calcium phosphate co-precipitation technique as described elsewhere (Francis-Lang *et al.*, 1992). Cells were harvested 48 h after transfection and the chloramphenicol acetyltransferase and luciferase activities were determined as described previously (Gorman *et al.*, 1982; de Wet *et al.*, 1987).

Extract preparation, band shift assays and DNase I footprinting

Nuclear extracts from FRTL-5 cells and total extracts from HeLa cells were prepared as previously described (Civitareale *et al.*, 1989). For gel shift assays, binding reactions were carried out in a buffer containing 20 mM Tris–HCl, pH 8.0, 75 mM KCl, 1 mM dithiothreitol (DTT), 1 mg/ml bovine serum albumin, 3 μg/reaction poly(dI·dC) and 10% glycerol. After 30 min incubation at room temperature, free DNA and DNA–protein complexes were resolved on a 5% polyacrylamide gel run in 0.5× Tris–borate–EDTA for 2–3 h at 4°C. The gel was dried and then exposed to X-ray film at –80°C overnight. In competition experiments, increasing molar excesses of competitor were added to a standard binding reaction mixture prior to addition of the labeled substrate and incubated at room temperature for 10 min. For DNase I footprinting assays, experiments were performed as previously described (Francis-Lang *et al.*, 1992).

RNA extraction and Northern blot analysis

Total RNA isolates were prepared by the acid guanidium thiocynate/phenol procedure (Chomczynski and Sacchi, 1987) and 30 μg of total RNA were electrophoresed through a 1% agarose gel containing 3.7% formaldehyde and 20 mM morpholinepropane sulfonic acid (MOPS) buffer. RNA was then blotted onto nylon membranes (Hybond-N) with 20× SSC and hybridization and washing were carried out according to Church and Gilbert (1984) with a 350 bp probe derived from the 3′ non-coding region of the cDNA labeled by random oligonucleotide priming.

Synthesis of RNA probes for in situ hybridization

For *in situ* hybridization experiments, a mouse TTF-2 sense RNA probe was synthetically produced using a 350 bp long fragment derived from the 3′-UTR of the cDNA subcloned in Bluescript. Mouse TTF-1 and Tg are the same probes as described in Lazzaro *et al.* (1991). Antisense strand transcription reactions with T3 or T7 polymerase were carried out in the presence of [^{35}S]CTP. The template was then degraded with RNase-free DNase (Pharmacia) and the labeled RNA was purified through a Sephadex G-50 column. *In situ* transcripts were progressively degraded by random alkaline hydrolysis to an average length of 150 nt. The probes were dissolved at a working concentration of 1×10^5 c.p.m./ml in the hybridization mix.

In situ hybridization

In situ hybridization was carried out as described by Wilkinson and Green with minor modifications. An aliquot of 30 μl of the appropriate probe in hybridization mix was added to each slide. Hybridization was carried out overnight at 55°C. The slides were then washed under stringent conditions (at 65°C in 2× SSC and 50% formamide) and treated with RNase to remove unhybridized and non-specifically bound probe. Autoradiography was performed with a Kodak NT/B2 emulsion. Exposure times were 5–12 days. After development, sections were stained with 0.02% toluidine blue and mounted in De Pex (Serva). Sections were examined and photographed using a Zeiss SV11 microscope with both dark and bright field illumination.

Acknowledgements

We thank Günther Schutz and Robert Costa for providing HNF3 clones, Mario De Felice, Caterina Missero and Paolo Macchia for reviewing the manuscript and for helpful discussion and Lucy Rowe for providing access to the BSS mapping panel. This work was supported by grants from the Progetto Finalizzato Applicazioni Cliniche della Ricerca Oncologica of the Consiglio Nazionale delle Ricerche, the Associazione Italiana per la Ricerca sul Cancro, the Commission of the European Communities (BIO2 CT93 0454) and National Institutes of Health grant GM18684.

References

Ambesi-Impiombato,F.S. and Coon,H.G. (1979) Thyroid cells in culture. *Int. Rev. Cytol.*, **10**, 163–172.

Aza-Blanc,P., Di Lauro,R. and Santisteban,P. (1993) Identification of a *cis*-regulatory element and a thyroid-specific nuclear factor mediating the hormonal regulation of rat thyroid peroxidase promoter activity. *Mol. Endocrinol.*, **7**, 1297–1306.

Bohinski,R.J., Di Lauro,R. and Whitsett,J.A. (1994) The lung surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol. Cell. Biol.*, **14**, 5671–5681.

Briata,P., Werken,R.V.D., Airoldi,I., Ilengo,C., Di Blas,E., Boncinelli,E. and Corte,G. (1995) Transcriptional repression by the human homeobox protein EVX1 in transfected mammalian cells. *J. Biol. Chem.*, **270**, 27695–27701.

Catron,K.M., Zhang,H., Marshall,S.A., Inostroza,J.A., Wilson,J.M. and Abate,C. (1995) Transcriptional repression by msx-1 does not require homeodomain DNA-binding sites. *Mol. Cell. Biol.*, **15**, 861–871.

Chidambaram,A. and Dean,M. (1996) Genetics of the nevoid basal cell carcinoma syndrome. *Adv. Cancer Res.*, **70**, 49–61.

Chomczynski,P. and Sacchi,N. (1987) Single step method of RNA isolation by acid guanidium thiocynate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

Church,G.M. and Gilbert,W. (1984) Genomic sequencing. *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.

Civitareale,D., Lonigro,R., Sinclair,A.J. and Di Lauro,R. (1989) A thyroid-specific nuclear protein essential for the tissue specific expression of the thyroglobulin promoter. *EMBO J.*, **8**, 2537–2542.

Clark,K.L., Halay,E.D., Lai,E. and Burley,S.K. (1993) Co-crystal structure of the HNF3/*fork head* DNA recognition motif resembles histone H5. *Nature*, **364**, 412–420.

Clevidence,D.E., Overdier,D.G., Tao,W., Qian,X., Pani,L., Lai,E. and Costa,R.H. (1993) Identification of nine tissue-specific transcription factors of the hepatocyte nuclear factor 3/forkhead DNA-binding domain family. *Proc. Natl Acad. Sci. USA*, **90**, 3948–3952.

Damante,G. and Di Lauro,R. (1994) Thyroid-specific gene expression. *Biochim. Biophys. Acta*, **1218**, 255–266.

De Felice,M., Damante,G., Zannini,M., Francis-Lang,H. and Di Lauro,R. (1995) Redundant domains contribute to the transcriptional activity of thyroid transcription factor 1 (TTF-1). *J. Biol. Chem.*, **270**, 26649–26656.

De Simone,V. and Cortese,R. (1991) Transcriptional regulation of liver-specific gene expression. *Curr. Opin. Cell Biol.*, **3**, 960–965.

de Wet,J.R., Wood,K.V., Deluca,M., Helinski,D.R. and Subramani,S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.*, **7**, 725–737.

Elkabes,S., Loh,Y.P., Nieburgs,A. and Wray,S. (1989) Prenatal ontogenesis of pro-opiomelanocortin in the mouse central nervous system and pituitary gland: an *in situ* hybridization and immunocytochemical study. *Dev. Brain Res.*, **46**, 85–95.

Ericson,L.E. and Frederiksson,G. (1990) Phylogeny and ontogeny of the thyroid gland. In Greer,M.A. (ed.), *The Thyroid Gland, Comprehensive Endocrinology.* Raven Press, New York, NY, pp. 1–35.

Falvo,J.V., Thanos,D. and Maniatis,T. (1995) Reversal of intrinsic DNA bends in the IFNβ gene enhancer by transcription factors and the architectural protein HMG I(Y). *Cell*, **83**, 1101–1111.

Francis-Lang,H., Price,M., Polycarpou-Schwarz,M. and Di Lauro,R. (1992) Cell-type expression of the rat thyroperoxidase promoter indicates common mechanisms for thyroid-specific gene expression. *Mol. Cell. Biol.*, **12**, 576–588.

Giese,K., Kingsley,C., Kirshner,J.R. and Grosschedl,R. (1995) Assembly and function of a TCRα enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein–protein interaction. *Genes Dev.*, **9**, 995–1008.

Gorman,C.M., Moffat,L.M. and Howard,B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.*, **2**, 1044–1051.

Gstaiger,M., Knoepfel,L., Georgiev,O., Schaffner,W. and Hovens,C.M. (1995) A B-cell coactivator of octamer binding transcription factor. *Nature*, **373**, 360–362.

Guazzi,S., Price,M., De Felice,M., Damante,G., Mattei,M.G. and Di Lauro,R. (1990) Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. *EMBO J.*, **9**, 3631–3639.

Hellqvist,M., Mahlapuu,M., Samuelsson,L., Enerback,S. and Carlsson,P. (1996) Differential activation of lung-specific genes by two forkhead proteins, FREAC-1 and FREAC-2. *J. Biol. Chem.*, **271**, 4482–4490.

Hermesz,E., Mackem,S. and Mahon,K.A. (1996) Rpx: a novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate and Rathke's pouch of the mouse embryo. *Development*, **122**, 41–52.

Herschlag,D. and Johnson,F.B. (1993) Synergism in transcriptional activation: a kinetic view. *Genes Dev.*, **7**, 173–9.

Holloway,J.M., Szeto,D.P., Scully,K.M., Glass,C.K. and Rosenfeld,M.G. (1995) Pit-1 binding to specific DNA sites as a monomer or dimer determines gene-specific use of a tyrosine-dependent synergy domain. *Genes Dev.*, **9**, 1992–2006.

Jonsson,J., Carlsson,L., Edlund,T. and Edlund,H. (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature*, **371**, 606–609.

Kaestner,K.H., Lee,K.-H., Schlondorff,J., Hiemisch,H., Monaghan,A.P. and Schutz,G. (1993) Six members of the mouse forkhead gene family are developmentally regulated. *Proc. Natl Acad. Sci. USA*, **90**, 7628–7631.

Kimura,S., Hara,Y., Pineau,T., Fernandez-Salguero,P., Fox,C.H., Ward,J.M. and Gonzalez,F. (1996) The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.*, **10**, 60–69.

Lamonerie,T., Tremblay,J.J., Lanctot,C., Therrien,M., Gauthier,Y. and Drouin,J. (1996) Ptx-1, a bicoid-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene. *Genes Dev.*, **10**, 1284–1295.

Lazzaro,D., Price,M., De Felice,M. and Di Lauro,R. (1991) The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the fetal brain. *Development*, **113**, 1093–1104.

Lin,S.-C., Li,S., Drolet,D.W. and Rosenfeld,M.G. (1994) Pituitary ontogeny of the snell dwarf mouse reveals Pit-1 independent and Pit-1 dependent origins of the thyrotrope. *Development*, **120**, 515–522.

Luo,Y., Fujii,H., Gerster,T. and Roeder,R.G. (1992) A novel B cell-derived coactivator potentiates the activation of immunoglobulin promoters by octamer-binding transcription factors. *Cell*, **71**, 231–241.

McPherson,C.E., Shim,E.-Y., Friedman,D.S. and Zaret,K.S. (1993) An active tissue-specific enhancer and bound transcription factors existing in a precisely positioned nucleosomal array. *Cell*, **75**, 387–398.

McPherson,C.E., Horowitz,R., Woodcock,C.L., Jiang,C. and Zaret,K.S. (1996) Nucleosome positioning properties of the albumin transcriptional enhancer. *Nucleic Acids Res.*, **24**, 397–404.

Mitchell,P.J. and Tjian,R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science*, **245**, 371–378.

Mock,B.A., Stoye,J., Spence,J., Jackson,I., Eppig,J.T., Fiedorek,F.T.,Jr and Neumann,P.E. (1996) Encyclopedia of the mouse genome V. Mouse chromosome 4. *Mamm. Genome*, **6** (special issue), S73–S96.

Monaghan,A.P., Kaestner,K.H., Grau,E. and Schultz,G. (1993) Postimplantation expression patterns indicate a role for the mouse *forkhead*/HNF-3 α, β, and γ genes in determination of the definitive endoderm, chordamesoderm and neurectoderm. *Development*, **119**, 567–578.

Murphy,M.J., Bell,G.I. and Friesen,H.G. (1987) Tissue distribution of insulin-like growth factor I and II messenger ribonucleic acid in the adult rat. *Endocrinology*, **120**, 1279.

Pierrou,S., Hellqvist,M., Samuelsson,L., Enerback,S. and Carlsson,P. (1994) Cloning and characterization of seven human forkhead proteins: binding site specificity and DNA bending. *EMBO J.*, **13**, 5002–5012.

Plachov,D., Chowdhury,K., Walther,C., Simon,D., Guenet,J.-L. and Gruss,P. (1990) Pax-8, a murine paired box gene expressed in the developing excretory system and thyroid gland. *Development*, **110**, 643–651.

Poleev,A., Fickenscher,H., Mundlos,S., Winterpacht,A., Zabel,B., Fidler,A., Gruss,P. and Plachov,D. (1992) PAX8, a human paired box gene: isolation and expression in developing thyroid, kidney and Wilms' tumors. *Development*, **116**, 611–623.

Rotwein,P., Pollock,K.M., Watson,M. and Milbrandt,J.D. (1987) Insulin-like growth factor gene expression during rat embryonic development. *Endocrinology*, **121**, 2141–2144.

Rowe,L., Nadeau,J., Turner,R., Frankel,W., Letts,V., Eppig,J., Ko,M., Thurston,S. and Birkenmeier,E. (1994) Maps from two interspecific backcross DNA panels available as a community genetic mapping. *Mamm. Genome*, **5**, 253–274.

Santisteban,P., Acebron,A., Polycarpou-Schwarz,M. and Di Lauro,R. (1992) Insulin and insulin-like growth factor 1 regulate a thyroid-specific nuclear protein that binds to the thyroglobulin promoter. *Mol. Endocrinol.*, **6**, 1310–1317.

Sato,K. and Di Lauro,R. (1996) Hepatocyte nuclear factor 3β participates in the transcriptional regulation of the thyroperoxidase promoter. *Biochem. Biophy. Res. Commun.*, **220**, 86–93.

Simmons,D.M., Voss,J.W., Ingraham,H.A., Holloway,J.M., Broide,R.S., Rosenfeld,M.G. and Swanson,L.W. (1990) Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes Dev.*, **4**, 695–711.

Sinclair,A.J., Lonigro,R., Civitareale,D., Ghibelli,L. and Di Lauro,R. (1990) The tissue-specific expression of the thyroglobulin gene requires interaction of thyroid-specific and ubiquitous factors. *Eur. J. Biochem.*, **193**, 311–318.

Strubin,M., Newell,J.W. and Matthias,P. (1995) OBF-1, a novel B-cell-specific coactivator that stimulates immunoglobulin promoter activity through association with octamer-binding proteins. *Cell*, **80**, 497–506.

Tanaka,K., Miura,N., Satokata,I., Miyamoto,I., Yoshida,M.C., Satoh,Y. and Kondo,S. (1990) Analysis of a human DNA excision repair gene involved in group A xeroderma pigmentosum and containing a zinc-finger domain. *Nature*, **348**, 73–76.

Thanos,D. and Maniatis,T. (1995) Virus induction of human IFNβ gene expression requires the assembly of an enhanceosome. *Cell*, **83**, 1091–1100.

Tjian,R. and Maniatis,T. (1994) Transcriptional activation: a complex puzzle with few easy pieces. *Cell*, **77**, 5–8.

Wilkinson,D.G. and Green,J. (1990) Postimplantation mouse embryos: a practical approach. Rickwood,D. and Cockroft,D.L. (eds). Oxford University Press, Oxford, pp. 155–171.

Zannini,M., Francis-Lang,H., Plachov,D. and Di Lauro,R. (1992) Pax-8, a paired domain-containing protein, binds to a sequence overlapping the recognition site of a homeodomain and activates transcription from two thyroid-specific promoters. *Mol. Cell. Biol.*, **12**, 4230–4241.

Zannini,S., De Felice,M., Sato,K. and Di Lauro,R. (1994) In Sizonenko,P.C., Aubert,M.L. and Vessalli,J.D. (eds), *Frontiers in Endocrinology*, Vol. 6, *Transcription Factors and Differentiation of Thyroid Follicular Cells*. Ares Serono Symposia Publications, Rome, pp. 79–87.

Received on November 11, 1996; revised on February 12, 1997