BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **220,** 86–93 (1996)
ARTICLE NO. 0361

Hepatocyte Nuclear Factor 3β Participates in the Transcriptional Regulation of the Thyroperoxidase Promoter

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Received January 26, 1996

The promoters of the thyroglobulin (Tg) and thyroperoxidase (TPO) genes both contain sequences that match the consensus recognized by the fork-head family of transcription factors. The fork-head recognition element in the TPO promoter is recognized by two proteins, one of which is the transcription factor HNF-3β, a member of the hepatocyte nuclear factor 3 (HNF-3) family, and the other is a thyroid-specific binding activity previously described as thyroid transcription factor 2 (TTF-2). Differently, only TTF-2 is capable of recognizing the fork-head recognition element in the thyroglobulin promoter. HNF-3β protein is present in cultured thyroid cells and in the adult thyroid gland, suggesting that it does indeed have a role in modulating thyroid-specific gene expression. The transcriptional activities of TPO promoter mutants impaired in either TTF-2 or HNF-3β binding suggest that both factors participate in regulating transcription from the TPO promoter.

Transcription factors can be grouped into classes, according to the structural motif used for recognition of specific DNA sequences (1). The fork-head gene family of transcription factors, also called FREAC or HNF-3 family (2–7), contains the fork-head domain, a hydrophilic, 110-amino-acid long, winged-helix (8–10) DNA-binding domain that has been identified in many genes and in a wide range of organisms (11–15). Fork-head containing genes have been grouped into classes base on their DNA binding specificity, that correlates with the primary structure of the fork-head domain (2, 10, 16, 17). Genetic studies, both in *Drosophila* and mice, clearly demonstrated that members of the fork head family play essential roles in pattern formation during embryogenesis (11, 12, 18–22). The HNF-3 subclass of fork-head genes includes three members, α, β and γ, that have been shown to be expressed in many endoderm derived cell types (19), where they have often been implicated in contributing to cell-type specific gene expression (18, 20–22).

Thyroglobulin (Tg) and thyroperoxidase (TPO) gene promoters have been demonstrated to be able to confer thyroid specific gene expression to heterologous genes both in tissue culture and, only for the Tg promoter, in transgenic animals (23–29). Full transcriptional activity of both promoters is dependent, amongst others, on the Z (TPO) and the K (Tg) region, that share sequence homology and have been suggested to be recognized by a similar transcription factor, called TTF-2 (23–26). On comparing the sequences of the K and Z regions with the DNA sequences recognized by known transcription factors, we found that both regions contain a DNA sequence motif very similar to the sequences recognized by proteins containing the fork-head DNA binding domain (2, 10, 16, 17), especially to the consensus proposed for the HNF-3 subfamily (10). This finding prompted us to speculate that TTF-2 might be a fork head containing protein, belonging to the HNF-3 subfamily.

This study shows that HNF-3β, a fork-head containing protein that plays an essential role during gastrulation (18–22) and that has also been involved in liver and lung-specific gene expression (30–33), is expressed in cultured thyroid cells and in adult thyroid gland. Furthermore, HNF-3β is

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Abbreviations: HNF-3, hepatocyte nuclear factor 3; FREAC, fork-head related activators; TTF-1, thyroid transcription factor 1; TTF-2, thyroid transcription factor 2; TPO, thyroperoxidase; Tg, thyroglobulin; CAT, chloramphenicol acetyltransferase; LUC, luciferase.

0006-291X/96 $18.00

found to bind to the fork-head recognition element of the TPO promoter but does not recognize the similar element of the Tg promoter. HNF-3β is different from the thyroid specific nuclear factor TTF-2, that binds to both K and Z regions. In the TPO promoter HNF-3β and TTF-2 show a functional redundancy, as they both appear to stimulate transcription through the same DNA sequence.

MATERIALS AND METHODS

Plasmids. Plasmid p420TPOL, containing the minimum TPO-promoter linked to the luciferase cistron, has been described previously (25). Mutants of the TPO promoter in the Z region (Zm, Zmu and Zmk) were created by the polymerase chain reaction by the overlap extension method (34) on the p420TPOL template. The amplified fragments were subcloned into p420TPOL replacing the wild-type promoter fragment. Plasmid pCMV3β, an expression vector for rat HNF-3β cDNA, was generously provided by Dr. R. H. Costa. Mouse HNF-3 plasmids for RNase protection analysis were a gift from Dr. G. Schütz. Sources of plasmids for transfection and other purposes were as described in previous paper (23–28).

Binding assays. Nuclear extracts were prepared from rat thyroid cell lines as previously described (23). Whole-cell extract (26) of HeLa cells transiently transfected with pCMV3β were used as a source of rat HNF-3β protein. The concentration of protein was determined with Bio Rad protein-assay reagent.

Gel retardation assays were performed with the incubated amount of extracts and 5′-end labeled probes, which were assembled in a reaction mixture, 20 mM Tris-HCl, pH 7.5, 200 mM KCl, 10% glycerol, 1 mg/ml of bovine serum albumin and 150 mg/ml of poly(dI-dC). After incubation for 30 minutes at room temperature, aliquots were loaded onto a 8% polyacrylamide gel that was run at 4°C. In competition experiments the competitor was preincubated with extracts for 10 min. prior to addition of the labeled probe. For supershift experiments, the aliquots were still incubated with 1 to 2 μl of antibodies on ice for 50 min. A HNF-3β specific antibody was raised against synthetic peptide corresponding to a divergent sequence (residues 311–324) of rat HNF-3β (4) and purified by an affinity column. Oligonucleotides used were:

Z, 5′ACAAATAATCAACAAAACGAATGG3′,
Zmd, 5′ACAAATACTAAACAAAGTGAATGG3′,
Zmu, 5′ACAAATAGAAAACAAACAGAATGG3′,
Zmud, 5′ACAAATAGAAAACAAAGTGAATGG3′,
Zmk, 5′ACGCAGAGAAAACAAAGTGAATGG3′,
K, 5′TGACTAGCAGAGAAAACAAAGTGA3′,
TTR, 5′TGACTAAGTCAATAATCAGAATCAG3′

DNase I footprinting was carried out on the minimum TPO promoter from the p420TPOL as described in (25). Interference analysis was essentially performed as described previously (25). For permanganate interference experiments, the labeled-single nucleotides were modified with 0.25 mM potassium permanganate in 30 mM Tris-HCl, pH 8.0, for 10 minutes at RT and annealed with the complementary strands as described by Truss, M. et al (35).

RNA analysis. Total RNA was prepared from a variety of adult rat tissues and cell lines by the acid guanidium thiocianate-phenol procedure (36). RNase protection analysis was performed according to (28) using labeled-antisense RNA probes: HNF-3α (positions 1165–1355), HNF-3β (positions 1169–1389) and HNF-3γ (positions 993–1330) of the mouse cDNA (6). The antisense were hybridized against total RNA fraction at 42°C in 80% formamide overnight. Excess probes were then removed by digestion with RNase ONE (Promega) and the protected probe fragments were analyzed on denaturing 6% polyacrylamide gel.

Cell culture and transfection. HeLa cells were grown in an atmosphere of CO_2/air (1:19) at 37°C in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. FRTL-5 cells, a cell line derived from normal rat thyroid, were grown in Coon's modified F-12 medium supplemented with 5% calf serum and six hormones as described in (37). Ha-*ras* and Ki-*ras* transformed FRTL-5 cells were characterized in reference (38).

For transient expression of rat HNF-3β, HeLa cells were plated at 1.5×10^6 cells in 100 mm dish 6 to 12 h prior to transfection. Plasmid pCMV3β (20 μg) coprecipitated with calcium phosphate was then added into the culture medium. Whole cell extracts containing over expressed HNF-3β were prepared 48 h after transfection.

For TPO mutation analysis in FRTL-5 cells, the cells were plated at 5×10^4 cells in 60 mm dish and incubated with the growing medium for 2 days. The medium was changed to DMEM containing 5% serum and six hormones 4 h prior to transfection. After being 1 h incubation with DNA (total 8 μg) and calcium phosphate precipitants, the cells were washed once with the serum-free medium and exposed to 15% glycerol in HEPES saline for 3 min. The cells were washed well again with the serum-free medium and maintained for 48 h in the growing medium before harvest. The chloramphenicol acetyltransferase (CAT) assays of pCMVCAT (2 μg) for variability in transfection between dishes were performed according to (39) and the signals obtained were quantified on a Instant Imager (Packard). The luciferase (LUC) activity of test constructs (6 μg) was determined according to (40).

RESULTS AND DISCUSSION

HNF-3β is expressed in cultured thyroid cells and in the adult thyroid gland. Studies carried out by *in situ* hybridization on mouse embryos have demonstrated that HNF-3α and β are expressed in the developing thyroid and their expression is maintained until day 12.5 and 14.5 post coitum, respectively (19). Hence, we studied the expression of HNF-3α and β in the adult thyroid and in normal and transformed thyroid cell lines by RNase protection analysis. The data shown in Fig. 1B revealed that mRNA of HNF-3β is present in adult thyroid and FRTL-5 cells, in addition to liver and lung as reported previously (4, 6, 33, 32). Neither HNF-3α nor HNF-3γ are detected in adult thyroid.

Transformation of FRTL-5 cells by several oncogenes results in loss or reduction of differentiation (26, 27, 38). To test whether also HNF-3β expression is sensitive to transformation, the presence of HNF-3β mRNA was assessed in Ha- and Ki-*ras* transformed FRTL-5. Fig. 1C shows that the mRNA encoding HNF-3β is severely reduced in a Ki-*ras* transformed cell line, whereas wild-type levels of HNF-3β mRNA were detected in Ha-*ras* transformed FRTL-5 cells. Interestingly, TTF-1, a transcription factor implicated in the establishment and maintenance of thyroid specific gene expression, shows a similar differential regulation by Ha- and Ki-*ras* (26, 41).

HNF-3β binds to the Z region of TPO promoter and has a DNA binding specificity different from TTF-2. To verify whether HNF-3β DNA binding activity is present in thyroid cells, we performed band shift experiments using FRTL-5 nuclear extracts and an oligonucleotide containing a well characterized HNF-3 binding site from the transthyretin promoter (TTR in Fig. 1A). The mobility of the complex obtained with the TTR oligonucleotide, was compared with that of the complexes formed with the K and Z oligonucleotides. At variance from what described before (25), we discovered that it was possible to detect two complexes with the Z oligonucleotides, C1 and C2 in Fig. 2A. Since C1 co-migrates with the complex formed by the same extracts with the TTR oligonucleotide, while C2 has the same mobility as the complex formed with the K oligonucleotide,

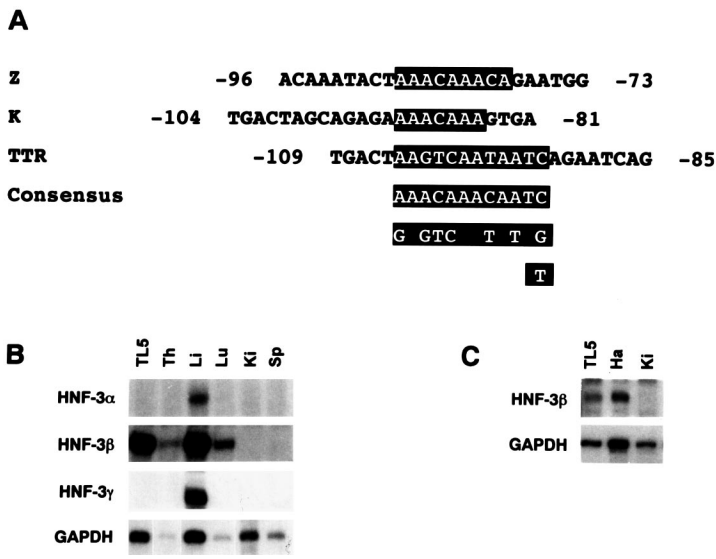

FIG. 1. HNF-3β is expressed in differentiated thyroid cells. (A) Putative matching region for a consensus DNA-binding sequence of HNF-3. Three oligonucleotides, Z from the TPO promoter, K from Tg (23, 25) and TTR from the transthyretin (3), are aligned with HNF-3 consensus binding sequence. (B) RNase protection analysis of HNF-3 in rat adult tissues and FRTL-5 cells. Total RNA (30 μg) from FRTL-5 cells (TL5), thyroid (Th), liver (Li), lung (Lu), kidney (Ki) and spleen (Sp) were hybridized with antisense RNA probes indicated to the left of the panel. (C) HNF-3β expression profile in transformed FRTL-5 cells. Ten μg of total RNAs from wild-type FRTL-5 cells (TL5) and FRTL-5 cells transformed with H-*ras* (Ha) and K-*ras* (Ki) were hybridized with RNA probes, HNF-3β and GADPH.

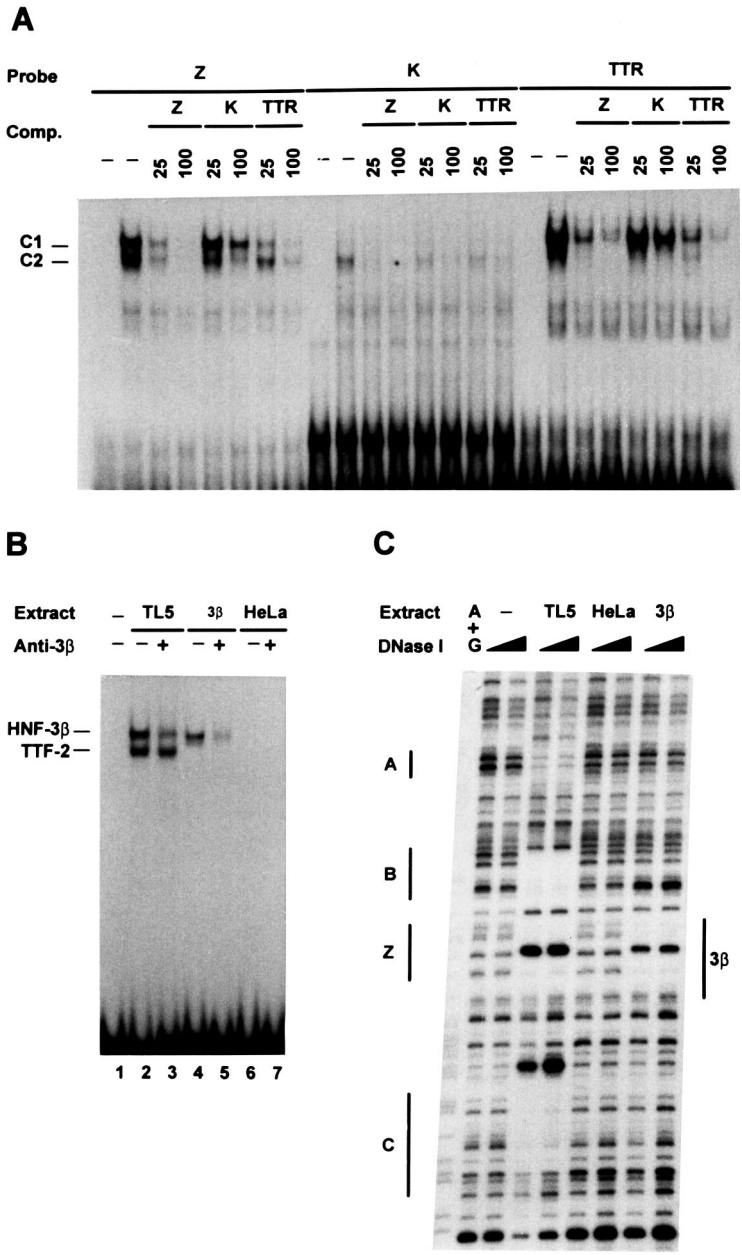

FIG. 2. Interaction of two thyroid nuclear proteins with both TPO and Tg promoters. (A) DNA binding activity of two nuclear proteins from FRTL-5 cells with three double-strand oligonucleotides; Z, K and TTR as described in the legend of Fig. 1A. Nuclear extracts (7.5 μg) were added in reaction mixtures for the gel retardation assay (see Materials and Methods) together with an excess amount of unlabeled competitor oligonucleotides (Comp.), incubated with labeled oligonucleotides (Probe), and subsequently separated in an 8% polyacrylamide gel. (B) Antibody recognition of HNF-3β in the complex of the Z oligonucleotide and a nuclear protein from FRTL-5 cells. Binding reactions were performed with three extracts; 7.5 μg of FRTL-5 nuclear extract (TL5), 1 μg of HNF-3β transfected and untransfected HeLa whole cell extract (3β and HeLa). Either 2 μl of an affinity-purified antibody against HNF-3β (lanes 3, 5 and 7) or phosphate buffer saline (lanes 2, 4 and 6) was added to the reaction mixtures. Anti-3β antibody disrupted the formation of the HNF-3β/probe complex. (C) DNase I footprint of the TPO promoter. TPO promoter was labeled at the Eco RI site (+75 relative to the transcription start site) within the polylinker of p420TPOL using polynucleotide kinase. Footprint reactions were performed as described in (25) with three extracts; 10 μg of FRTL-5 nuclear extracts (TL5), 4 μg of HNF-3β overexpressed HeLa cell extracts (3β) and 4 μg normal HeLa cell extracts (HeLa). Maxam-Gilbert sequencing reaction was indicated as A+G. Protections given by nuclear proteins from FRTL-5 cells are depicted as lines at the left. The protected region of HNF-3β is similar to the Z-region of FRTL-5 nuclear proteins.

we hypothesized that HNF-3β is responsible for complex C1 and TTF-2 for C2. To verify such an hypothesis, we attempted supershift experiments with extracts from either FRTL-5 or HeLa cells transfected with an HNF-3β expression vector (Fig. 2B). Addition of an anti-HNF-3β affinity purified antibody in binding reactions caused a selective reduction of the low mobility complex, indicating that HNF-3β is a component of the complex C1. DNase I footprinting, carried out on the proximal TPO-promoter in p420TPOL (Fig. 2C) with nuclear extracts from either FRTL-5 or HNF-3β expressing HeLa cells show a very similar pattern of protection in the Z region (compare TL5 and 3β in Fig. 2C), thus providing additional evidence that thyroid cells contain HNF-3β protein that is capable of binding to the TPO promoter. Since no footprint was observed in the K region of the Tg-promoter with HNF-3β expressing HeLa cell extracts, we conclude that TTF-2, the thyroid protein that binds to this region of the Tg promoter, is not HNF-3β (data not shown).

Contacts established by HNF-3β with the Z region of TPO promoter. Nuclear extracts from HNF-3β expressing HeLa cells were used to determine the nucleotides important for recognition by HNF-3β. The 5′-end labeled upper- or lower-strand of Z oligonucleotide, modified with either

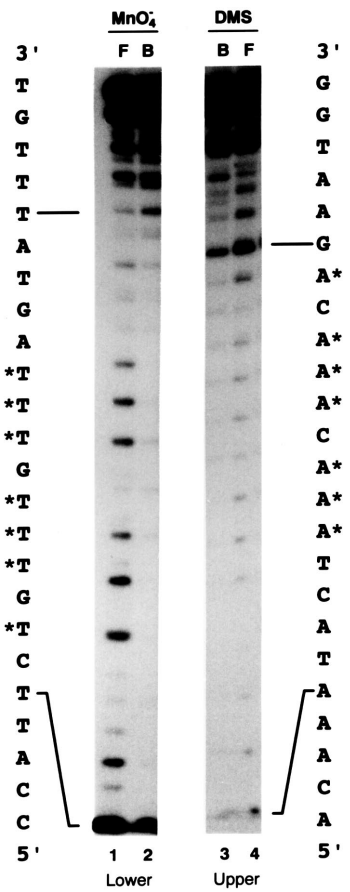

FIG. 3. Methylation- and permanganate-interference analysis of HNF-3β with the Z oligonucleotide in the TPO promoter. 5′-end-labeled upper and lower Z-oligonucleotide were modified with dimethyl sulfate (DMS) and permanganate (MnO_4^-), respectively. Six μg of HNF-3β fraction extracted from HeLa cells was incubated with each modified oligonucleotide for a preparative gel. The bands corresponding to bound complex (B) and free DNA (F) were eluted from the gel and cleaved at the modified bases. The cleavage products were resolved by 20% denaturing gels. Both sequences of the Z-oligonucleotides are indicated at each side of the panel. Asterisks indicate the base whose modification reduces the proportion of DNA in the bound fraction.

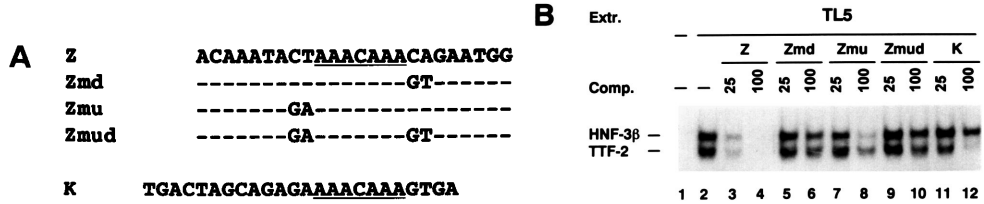

FIG. 4. Different effects of mutations on the binding of HNF-3β and TTF-2 to the TPO promoter Z region. (A) For the comparison of relative binding activity between HNF-3β and TTF-2 to the Z region, six different mutations are introduced in either Z or K oligonucleotide. A putative sequence common to binding sites of both proteins is underlined. (B) The competitive binding analysis on the Z oligonucleotide with FRTL-5 nuclear extracts. Reaction mixtures for the gel retardation assay were assembled as described in the legend of Fig. 2A. The position corresponding to both bands for HNF-3β and TTF-2 is indicated at the left of the panel.

dimethyl sulfate or permanganate, was used in band-shift interference experiments to reveal purines or thymidines involved in contacts with HNF-3β. Fig. 3 shows the results of such experiments and the pattern of interference underlines the relevance of the motif 3′-TTTGTTTGT-5′. This sequence is very similar to HNF-3 binding sites located in the albumin (TGTTTGTTCTC) or transferrin (CTTTGTTTGCTTC) enhancer (42, 43). Similar patterns of interference were obtained with both

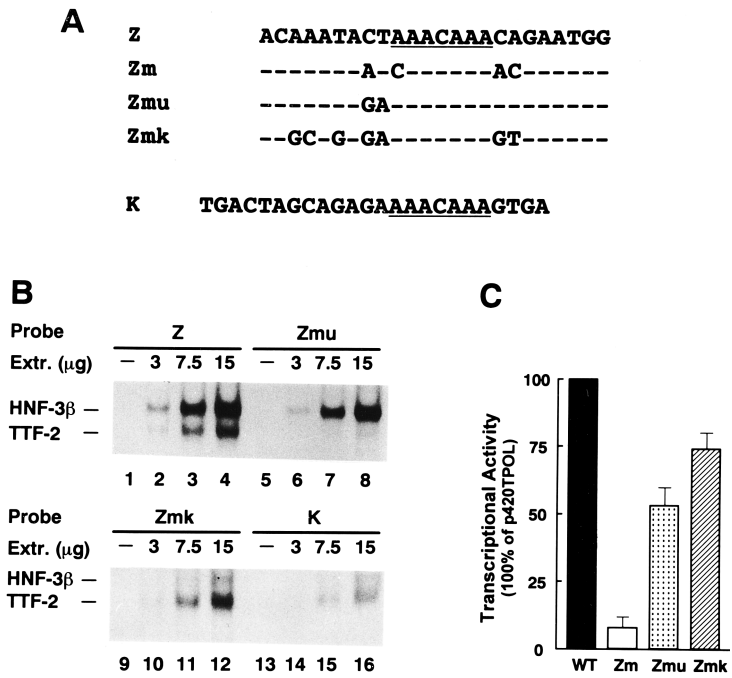

FIG. 5. Interplay of HNF-3β and TTF-2 on the TPO promoter. (A) Alignment of three nucleotides on the TPO-promoter Z-region significantly reduce the binding activity of HNF-3β and/or TTF-2. Underline indicates a putative sequence common to binding sites of both proteins. (B) Mutation of Z oligonucleotides eliminates factor binding *in vitro.* The gel retardation assay was performed with the indicated amount of nuclear extracts from FRTL-5 cells together with either the wild-type or the mutated Z oligonucleotide. The same intensity of labeled probes were added in the binding mixture. (C) Relative transcriptional activities between the wild-type and the mutated TPO promoter in FRTL-5 cells. Six μg of TPO promoter constructs, wild-type (WT) and mutants within the Z sequence (Zm, Zmu and Zmk) were transiently transfected with 2 μg of pCMVCAT as an internal control. Luciferase reporter gene activity was determined from extracts expression equivalent amount of CAT activity and expressed as percentages of WT activity (100%). The results shown are average values +/− S.E. of more than five independent experiments.

the C1 and C2 complexes obtained with the Z oligonucleotide when using FRTL-5 nuclear extracts, suggesting that HNF-3β and TTF-2 interact in similar manner with this sequence (data not shown).

To investigate the basis of differential recognition of Z (recognized by both TTF-2 and HNF-3β) and K oligonucleotide (only bound by TTF-2) (Fig. 2A), we used oligonucleotides that were mutated around the core sequence 5′-AAACAAA-3′ (Fig. 4A) in competition experiments. When the oligonucleotide Z is incubated with FRTL-5 nuclear proteins, C1 (HNF-3β/Z complex) and C2(TTF-2/Z complex) are formed in an approximately equal amount (Fig. 4B, lane 2) and they are equally competed by an excess of cold Z oligonucleotide. As expected, an excess of the K-oligonucleotide in the binding mixture competes only for TTF-2 binding (see lanes 11, 12). Z-oligonucleotide mutants show a differential behavior. Zmu competes very poorly for TTF-2 binding, while it is still able to compete for HNF-3b binding, albeit with a slightly reduced efficiency (compare lanes 2, 7 and 8); the differential effect of the Zmu mutation is clearly illustrated also by the experiment shown in Fig. 5B. At variance from Zmu, Zmd and Zmud show a substantial decrease in affinity for both proteins (compare lanes 2, 5, 6, 9 and 10). These results suggest that the CT sequence on the 5′ side of the core motif is important for HNF-3β binding to the Z-oligonucleotide, while the CA on the 3′ side is equally important for HNF-3β and TTF-2.

Both HNF-3β and TTF-2 participate in regulation of TPO-promoter transcriptional activity. To investigate whether HNF-3β and/or TTF-2 contribute to the activity of the TPO promoter, we used three mutated Z oligonucleotides, Zm, Zmu and Zmk (Fig. 5A). The Zm oligonucleotide has already been reported not to be recognized by any protein in extracts of thyroid cells (25) and we have confirmed that Zm cannot bind to either HNF-3β or TTF-2 (data not shown). Zmu has a 2 bp mutation upstream of the core sequence in the Z sequence and shows selective inhibition of TTF-2 binding (Figs. 4 and 5B). Zmk is a Z oligonucleotide that has been made identical to the K sequence, according to the alignment shown in Fig. 5A and, as expected, binds TTF-2 but not HNF-3β. The transcriptional activities of TPO promoter mutants carrying the mutations Zm, Zmu and Zmk show that while the Zm mutant has a very much reduced activity, in agreement with (25), both Zmu and Zmk showed significant transcriptional activities. Taken together, these data strongly suggest that both HNF-3β and TTF-2 could contribute to full promoter activity of the TPO-promoter through binding to the Z-region.

In conclusion, the present study provides concrete evidence that HNF-3β, a fork-head domain containing protein, participates in thyroid specific TPO expression by binding to its promoter Z-region. Such a role is redundant with that of TTF-2, a thyroid specific transcription factor which we have recently cloned and demonstrated, as expected on the basis of its DNA binding specificity, that it also belongs to the fork-head family of transcription factors.

ACKNOWLEDGMENTS

We are rateful to Dr. R. H. Costa for the generous supply of rat HNF-3β expression plasmids and Dr. G. Schütz for the generous gift of mouse HNF-3 plasmids for RNase protection analysis. We also thank Dr. Elio Biffali for the synthesis of oligonucleotides. K.S. was supported by a fellowship from the Italian–Japanese Biological Association and by a fellowship from Associazione Italiana per la Ricerca sul Canero. This work was supported by grants from the Progetto finalizzato Applicazioni Cliniche della Ricerca Oncologica of Consiglio Nazionale delle Ricerche, the Associazione Italiana per la Ricerca sul Canero and the Commission of the European Communities (BIO2 CT 930454).

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