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A thyroid-specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter

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A rat thyroglobulin promoter fragment, capable of directing thyroid-specific transcription, binds at least three different factors, TTF-1, TTF-2 and UFA, which are all present in nuclear extracts of the differentiated rat thyroid cell line FRTL-5. TTF-1 and TTF-2 are FRTL-5 specific, as demonstrated by their absence in nuclear extracts prepared from cell lines that do not express any thyroid-differentiated function, while UFA is present in all cell lines tested. TTF-1 has been extensively purified. It binds to the rat throyglobulin promoter at three diffrerent sites which share sequence homology. Mutations in two of the three sites decrease both binding of TTF-1 *in vitro* and promoter function *in vivo*. This suggests that the tissue-specific expression of the thyroglobulin genes is mediated, at least in part, by the presence of a transcription factor exclusively in thyroid cells.

Key words: rat thyroglobulin promoter/thyroid specific expression/transcription factor/FRTL-5/TTF-1/TTF-2/UFA

Introduction

The observation that the tissue-specific expression of some genes can be reproduced in tissue culture by the reintroduction of cloned genes in the appropriate cell types (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983; Walker *et al.*, 1983) has provided the opportunity to define DNA sequence elements that, by the interaction with an appropriate cellular environment, are, at least in part, responsible for the observed selective expression. This has in turn led to the identification of tissue-specific factors which interact with the *cis*-acting DNA elements, possibily to activate transcription in the appropriate cell type (Lichtensteiner *et al.*, 1987; Lefevre *et al.*, 1987; Lenardo *et al.*, 1987; Monaci *et al.*, 1988). In a few cases, transcriptional activation of a transfected tissue-specific promoter in non-permissive cell lines has been obtained upon cotransfection of a cloned cDNA for the cognate factor, demonstrating a direct role for such factors in the cell-type-specific induction of transcription (Ingraham *et al.*, 1988; Muller *et al.*, 1988).

The follicular cells of the thyroid gland express a complex differentiated phenotype that is finalized to the regulated biosynthesis and secretion of thyroid hormones. To this end the follicular cells are organized in a tridimensional structure (the thyroid follicle), actively concentrate iodine from the bloodstream through a specific channel, and produce a peroxidase that catalyses both the iodination and the coupling of specific tyrosine residues within the thyroglobulin molecule (Salvatore and Edelhoc, 1973). The rat thyroid cell line FRTL-5 (Ambesi-Impiombato *et al.*, 1980), which expresses in tissue culture most of the differentiated thyroid phenotype, except the typical follicular organization observed *in vivo*, provides a system whereby to study the biochemical mechanisms leading to the maintenance of the differentiated thyroid phenotype.

We are interested in defining the biochemical steps leading to the maintenance of the differentiated thyroid phenotype using, as an experimental system, the FRTL-5 cell line. The best characterized thyroid-specific function, which is also the first one that appears during vertebrate thyroid development (Kawaoi and Tsuneda, 1985) is thyroglobulin, the glycoprotein substrate for thyroid hormone biosynthesis (Edelhoc and Robbins, 1986). Previous studies indicated that a DNA fragment derived from the thyroglobulin gene and containing the transcription start site (Musti *et al.*, 1986), was able to function as a promoter if introduced in the differentiated thyroid cell line FRTL-5, whereas no transcription could be detected after transfection of the same fragment into a number of cell lines that do not express the endogenous thyroglobulin gene. The region $-168/+36$ of the thyroglobulin gene was demonstrated to be necessary and sufficient in order to obtain FRTL-5-specific expression. In addition, preliminary protein-binding studies showed the presence in FRTL-5 nuclear extracts of a factor binding to the $-50/-70$ region of the promoter (Musti *et al.*, 1987). We report in this paper an extensive characterization of the proteins present in crude nuclear extracts from FRTL-5 cells and in control non-thyroid cells, which can bind to the thyroglobulin promoter. At least three different activities, TTF-1, TTF-2 and UFA, which together recognize five sites, could be identified. One of the activities, TTF-1, has been extensively purified. Among the cell lines and tissues tested, we could detect TTF-1 activity exclusively in FRTL-5 cells and in thyroid tissue. It binds to three sites within the thyroglobulin promoter which share sequence homology. The effect of mutations in the binding sites for TTF-1 suggest that it is a positive factor which mediates thyroid-specific expression of the promoter.

Results

Several binding activities are present in crude nuclear extract of FRTL-5 cells

Nuclear extracts from FRTL-5 cells protect five regions of the thyroglobulin promoter from DNase I digestion. The protected regions are marked A′, A, B, K and C in Figure 1. The A′ footprint is located outside of the region shown to be sufficient for maximal expression of the promoter (Musti *et al.*, 1987), and therefore has not been studied further. Close inspection of the DNA sequence in the protected regions reveals a common sequence in regions A,

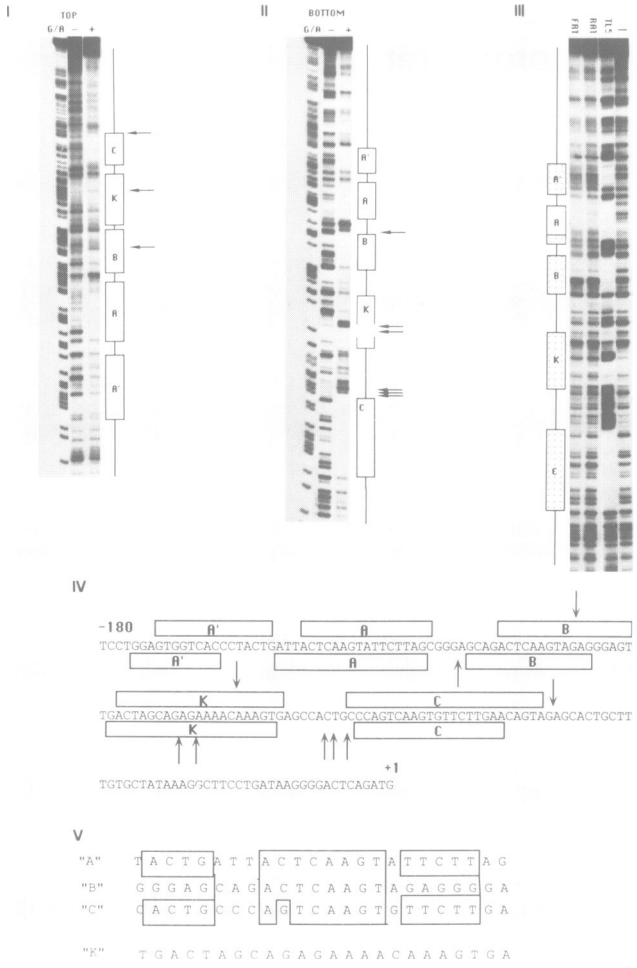

Fig. 1. Footprints on the rat thyroglobulin promoter. (**A**) *Bam*HI/*Hin*dIII DNA fragment, extending from −284 to +36 and derived from the plasmid 5′-41 (Musti *et al.*, 1987) was used as substrate in all cases. The fragment was labelled with [γ-^{32}P]ATP and polynucleotide kinase at either the *Hin*dIII (bottom strand) or at the *Bam*HI (top strand) site and was then digested with DNase I in the presence (+) or absence (−) of FRTL-5 nuclear extract (**panels I and II**). In **panel III**, the cell lines used as a source of nuclear extract are indicated at the top of the respective lane. G + A sequencing reactions (G/A), run on the same gel alongside the footprints, were used as size markers. Boxes and arrows indicate the protected regions and the hypersensitive sites respectively. The dotted boxes in **panel III** indicate footprints which are specific to FRTL-5. In **panel IV** the footprinting information is aligned on the thyroglobulin promoter sequence. **Panel V** shows the sequence selected to design oligonucleotides used in the band-shift experiments. Nucleotides which are identical between the A, B and C oligonucleotides are boxed.

B and C, while the DNA sequence of the K region is clearly different (Figure 1, panel IV). Nuclear extracts from the cell lines FRT (Avvedimento *et al.*, 1985) and Rat-1 (Botchan *et al.*, 1976), which do not express the endogenous thyroglobulin gene, protect only the A region. The footprint is slightly smaller than the one obtained with FRTL-5 extracts but is centered on the same sequence (Figure 1, panel III).

On the basis of the footprinting information, we designed four double-stranded oligonucleotides (Figure 1, panel V), containing the protected sequences, which were used as substrates to detect specific DNA−protein complexes in band-shift assay. The results of this experiment (Figure 2) demonstrate that the activities binding to oligonucleotides B, C and K are present only in FRTL-5 nuclear extracts.

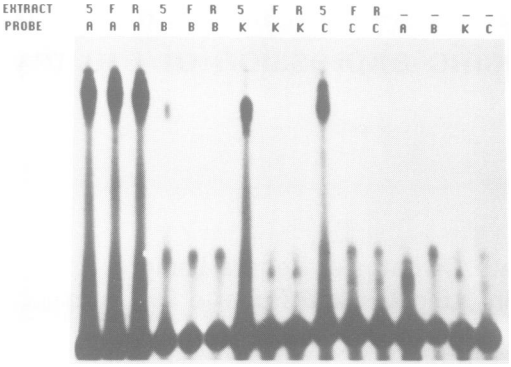

Fig. 2. Band-shift experiment using nuclear extracts derived from the different cell lines. The indicated oligonucleotide probe was incubated with nuclear extracts from FRTL-5(5), FRT(F) or Rat-1(R) and run on a 6% gel as described in Materials and methods.

Extract titration experiments demonstrate that while we can still detect a specific complex with oligonucleotides C and K using 0.5 μg of FRTL-5 nuclear proteins, we are unable to detect any complex using up to 20 times more protein of the 'non thyroid' cell extracts (data not shown). An activity capable of forming a complex with the A oligonucleotide is found instead also in nuclear extracts of FRT and Rat1 cells.

A thyroid-specific protein binds to the repeated motif A, B, C. An additional non-thyroid-specific protein binds to the motif A

The sequences protected by FRTL-5 nuclear extracts in the A, B and C region of the promoter show an evident sequence homology, being most striking between A and C. Nonetheless, while the B and C regions are protected by an activity exclusively present in the FRTL-5 cells the A region seems to bind a protein that is present also in FRT and Rat-1 cells. In order to analyse this observation further, we performed band-shift competition experiments. The oligonucleotides A, B, C and K were separately labelled and incubated with FRTL-5 nuclear extracts, with or without an excess of each of one of them, non-labelled, as competitor. The results of such an experiment demonstrate that the complex formed with the C oligonucleoitide can be competed by an excess of A, B or C oligonucleotides, while an excess of the K oligonucleotide has no effect on complex formation. The same result is obtained when the labelled B oligonucleotide is used as a substrate. This group of data suggest that the same, FRTL-5 specific (Figure 2) activity, which we call TTF-1, can form a complex with the B and C oligonucleotides and, at the same time, it can recognize the DNA sequence of the A oligonucleotide. On the other hand, a different activity must be responsible for the complex formed with the A oligonucleotide, as indicated both by its ubiquitous distribution (Figure 2) and by the specific competition pattern which indicates that only an excess of cold A oligonucleotide is effective in inhibiting complex formation (Figure 3). We explain this apparent contradiction by postulating the presence of at least two binding sites on the oligonucelotide A, one for an activity, which we call UFA (Ubiquitous Factor A), present in all three cell lines tested, and the second for TTF-1. In the band-shift assay with the oligonucleotide A we can only detect the complex formed with UFA, as judged by the inability of both the B

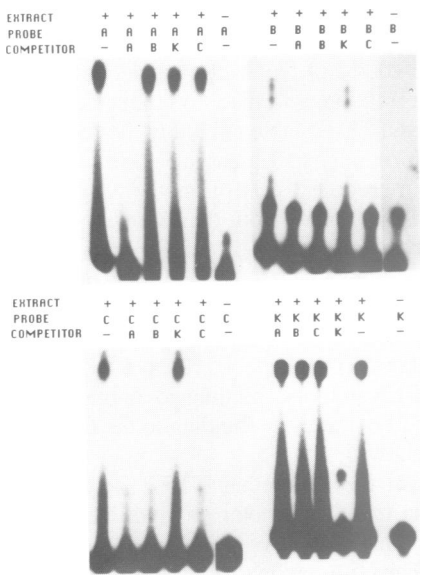

Fig. 3. Band-shift competition experiments. The indicated oligonucleotide probe was incubated with FRTL-5 nuclear extract in the presence or absence of 1000-fold molar excess of different competitors and electrophoresed on a 6% gel as described in Materials and methods.

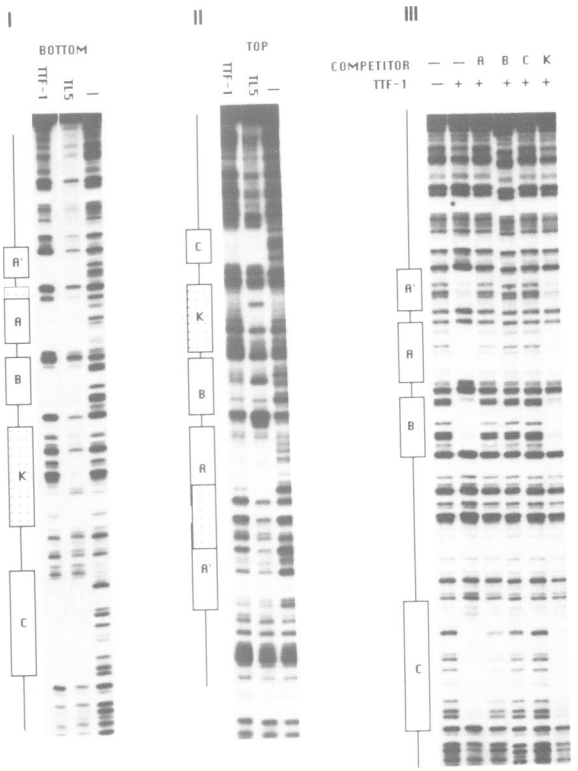

Fig. 4. Footprints using purified TTF-1. **Panels I** and **II**: comparison of the footprints obtained with crude extract and purified TTF-1. Top (**panel I**) and bottom (**panel II**) strands are as in Figure 1. Dotted boxes indicate the regions where the footprints are different. **Panel III**: competition with different oligonucleotides on the TTF-1 footprints. Only the bottom strand probe was used. Competing oligonucleotides were added at 12 ng/reaction.

and C oligonucleotides to compete for complex formation. The inability to detect a complex between TTF-1 and the oligonucleotide A is probably due to the low affinity of TTF-1 for the A sequence and by the consequent preponderant amount of the UFA/A complex which may obscure a small amount of TTF-1/A. The oligonucleotide corresponding to the K region clearly binds a different, thyroid-specific protein (Figure 2), since it does not compete with any of the other oligonucleotides in complex formation and, conversely, none of the other oligonucleotides compete for K complex formation (Figure 3).

TTF-1 can bind to the A region in the absence of UFA

In order to gain direct evidence for the binding of TTF-1 to the A region of the thyroglobulin promoter we used a purified preparation in footprinting experiments. TTF-1 has been purified from calf thyroids by a combination of conventional and sequence-specific DNA affinity chromatography (data not shown). The purified TTF-1 is devoid of TTF-2 activity. As shown in Figure 4, panel 1, TTF-1 is not able to protect the K region from DNase I digestion. In addition, the purified TTF-1 is devoid of UFA activity. On the basis of the experiment shown in Figure 3 we have defined the UFA DNA binding activity as the one able to form with the A oligonucleotide a complex which could not be competed by the B and C oligonucleotides. Conversely, the footprints obtained with TTF-1 on the A, B and C regions, can all be simultaneously competed by each of the A, B and C oligonucleotides, while the addition of the K oligonucleotide to the footprinting reaction has no effect (Figure 4, panel II), so proving that TTF-1 is not contaminated by UFA and that by itself it is able to bind to the A as well as to the B and C regions of the promoter. It should be noted that while the footprints obtained with purified TTF-1 in the B and C regions are identical to the ones obtained with crude FRTL-5 extracts, in the A region the two footprints show some differences near the 5′ border of the protected area.

Effect of mutations at the TTF-1 binding sites

As shown in Figure 1 the sequence of regions A, B and C is highly homologous, mostly in the central eight nucleotides. In order to evaluate the role of TTF-1 binding in the function of the thyroglobulin promoter, we constructed, by site-directed mutagenesis, three similar mutants in the A, B and C sites (A_{core}, B_{core}, C_{core}). In each mutant the same DNA sequence (CAGACCTG) replaces the conserved central octanucleotide sequence. The mutant DNAs were then introduced into FRTL-5 cells by calcium-phosphate-mediated transfection along with a CMV−luciferase construct (a generous gift of Ulrich Deuschle) that was used as an internal control to correct for variability in transfection efficiency. Mutations in the A and C regions have a considerably reduced promoter activity, while the B region mutant transcribes at almost wild-type levels (Figure 5, panel I). Footprints on the three mutant templates show that in all cases the mutation did reduce the binding of TTF-1 (Figure 5, panel II). In addition, band-shift competition experiments (Figure 5, panel III) demonstrate that the A_{core} mutation also abolished UFA binding to the A oligonucleotide. We deduce from this data that the binding of TTF-1 at the C region is essential for promoter activity, while the interaction at the B region appears to be largely dispensable. The greatly reduced transcriptional activity of plasmid templates containing an A_{core} mutant of the thyroglobulin promoter indicate that also

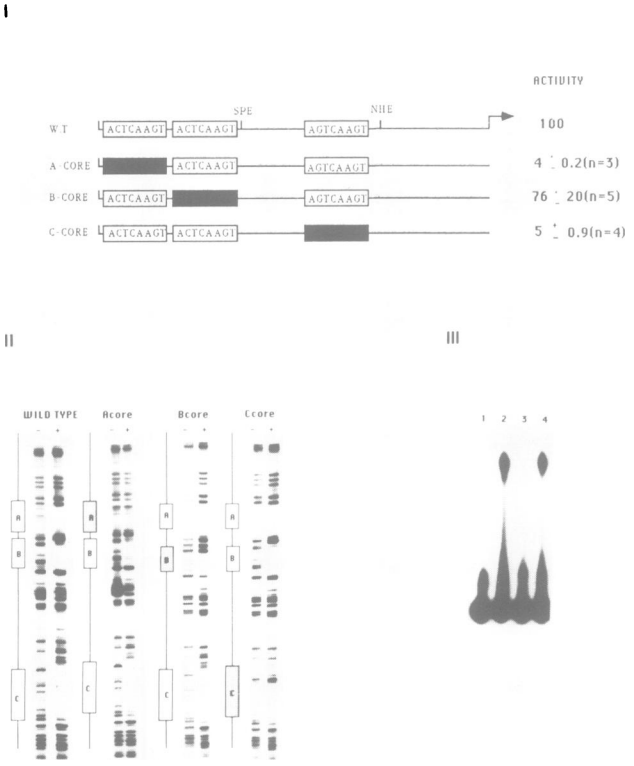

Fig. 5. Panel I: *in vivo* activity of mutant promoters. The three binding sites for TTF-1 are indicated with boxes displaying the sequence conserved among the three sites. In each mutant a black box indicates the mutated area, where the conserved region is replaced by the sequence 5′-CAGACCTG-3′. The strength of mutant promoter is expressed relative to the wild type, whose activity is arbitrarily set to 100. **Panel II**: TTF-1 footprints on mutated promoters. A similar fragment derived from the wild type and from the three 'core' mutants was 5′ end labelled in the bottom strand and footprinted with purified TTF-1. The DNA fragment used contains the thyroglobulin promoter sequence from −168 to +36 and extends into neighbouring plasmid sequence on the 5′ side of the promoter (see Materials and methods). Open boxes indicate protected regions, dotted ones show the mutated regions. **Panel III**: band shift competition with the A_{core} mutant oligonucleotide. The labelled oligonucleotide A was incubated with (**lanes 2–4**) or without FRL-5 nuclear extact (**lane 1**) in the presence of a 2000-fold molar excess of either cold oligonucleotide A (**lane 3**) or of the same amount of oligonucleotide A containing the A_{core} mutation (**lane 4**). No competitor control is shown in lane 2.

that region is essential for promoter activity, but, because both TTF-1 and UFA show no binding to the A region of this mutant, we cannot conclude whether either one of them or both are required in that region for full promoter activity.

Discussion

In the present study we demonstrate that the thyroid-specific protein TTF-1 can recognize three similar sequences present in the thyroglobulin promoter (regions A, B and C). We also prove that a mutant in the C region (C_{core}, Figure 5), that abolishes TTF-1 binding, greatly reduces promoter function, so supporting an important role of TTF-1 in maintaining high rate of transcription from the thyroglobulin promoter in thyroid cells. Also the A region has an important role in thyroglobulin promoter function, as demonstrated by the greatly reduced promoter strength in the A_{core} mutation (Figure 5). We believe that at least two proteins are able to make sequence-specific contacts in that region: TTF-1 and an ubiquitous protein (UFA). The ability of TTF-1 to recognize the A region comes from several observations, chiefly the ability of the A oligonucleotide to compete for complex formation with the C oligonucleotide and the fact that purified TTF-1 is able to bind to the A region, as shown by the footprinting experiment in Figure 4. Evidence for the existence in crude extracts of another protein able to bind to the A region comes from the following experiments: (i) while the complex formed by the A oligonucleotide in crude extract is insensitive to competition by the B and C oligonucleotides, such a competition is effective on the complex formed between the A sequence and TTF-1 (Figure 5, panel II); (ii) heated (65°C × 5′) FRTL-5 extracts conserve UFA activity (binding to A oligonucleotide) but they loose TTF-1 activity (binding to the C oligonucleotide) (data not shown).

We cannot prove whether TTF-1 or UFA or both are contributing to full promoter strength by binding at the A site. Both bindings are greatly reduced by the A_{core} mutation. The observation that the UFA protein has a higher affinity for the A site than TTF-1, as indicated by the preferential binding of the A oligonucleotide to the UFA protein in band shift experiments (Figures 2 and 3), would suggest that it is UFA that occupies the A site *in vivo*. On the other hand, one must be careful to transfer the observation made in a static binding assay to a dynamic situation, such as an actively transcribed promoter. In the case of α-1 antitrypsin, for example, two proteins can bind at the same site in a mutually exclusive fashion, LFB1 and LFB2. While in the footprinting assay LFB2 occupies the site, it is LFB1 that is required for transcription, while LFB2 has no clear role in promoter activity (Monaci *et al.*, 1988). In favour of a role for TTF-1 binding at the A site, is the conservation in promoter organization between rat, human and bovine thyroglobulin genes. Interestingly, only a duplication of a TTF-1-like binding site is present in the human and bovine promoter, equivalent in position to the rat A and C site, the third site B being present only in the rat (Civitareale *et al.*, 1987) but, as demonstrated in this paper, this site has no apparent role in promoter function. Preliminary results of additional mutations in the A region indicate that both UFA and TTF-1 may be required to interact in the A region for full promoter strength (A.J.Sinclair and R.Di.Lauro, unpublished observations), even if the overlap between the binding sites would suggest that their binding is mutually exclusive. A similar case occurs in the mouse albumin gene promoter (Lichtensteiner *et al.*, 1987), where the mutually exclusive interactions of a liver-specific and an ubiquitous factor at two overlapping binding sites are both necessary for promoter function. Alternatively, TTF-1 and UFA may be binding at the same time to the same area. This could be possible if the two proteins make sequence-specific contacts on the two opposite sides of the DNA helix.

The thyroid-specific protein binding to the K region (TTF-2, Figure 2) does not seem to play such an important role as TTF-1 in thyroglobulin promoter function, since mutations in this region reduce transcription, but do not have the drastic effect on promoter activity observed with the A_{core} or C_{core} mutations (A.J.Sinclair and R.Di Lauro, unpublished observations). The role of the K region interaction(s) could be to establish rather than maintain thyroid-specific transcription, as seems to be the case for

NF-kB in B cells (Atchison and Perry, 1987). Another possibility is that TTF-2 could be involved in other regulatory pathways, like the TSH (Avvedimento *et al.*, 1984; van Heuverswin *et al.*, 1984) or insulin (Santisteban *et al.*, 1987) induction of thyroglobulin transcription. Alternatively the thyroid-specific protein(s) interacting at K could be a redundant signal for thyroglobulin transcription and possibly a relevant element in regulating transcription of other thyroid-specific genes.

The structural organization of the thyroglobulin promoter is reminiscent of the rat prolactin and growth hormone gene promoters (Lefevre *et al.*, 1987; Nelson *et al.*, 1988), where several copies of a binding site for the same pituitary-specific proteins have been described. Also in those promoters a hierarchy is observed among the different binding sites. Interestingly, not always weaker binding signifies a less important role in promoter strength. In the thyroglobulin promoter the site with the highest affinity seems to be the closest one to the start point of transcription (site C), as judged by the intensity of the signal obtained in band-shift experiments (Figure 2). We have never observed a preferential occupancy of site C compared with A and B in footprinting experiments, when the three sites are present on the same DNA molecule. On the other hand, strong cooperative effects among the three binding regions seem to be ruled out by the observation that mutations at either of the three sites do not clearly change the binding affinity of the non-mutated sites for TTF-1 (data not shown). Whether the signals that we describe in this paper are the unique determinants for thyroid gene expression cannot be stated at this point. Nonetheless, we do know that at least one of the TTF-1 binding sites plays an essential role in promoter function, that TTF-1 activity is restricted to FRTL-5 cells, and that it disappears when the differentiated phenotype is extinguished by transformation (Avvedimento *et al.*, 1988). This evidence suggests that TTF-1 is a very important molecule for the maintenance of the differentiated thyroid phenotype. We are currently designing TTF-1-specific probes from the peptide sequence of the purified protein (S.Guazzi, D.Civitareale and R.Di Lauro, unpublished), which allow us to define the regulatory mechanisms which restrict TTF-1 activity to the thyroid tissue.

We have shown previously that FRTL-5 cells, when transformed by Kirsten Murine sarcoma virus lose their differentiated phenotype and have no detectable TTF-1 activity in their nuclear extract. The demonstration that TTF-1 is an essential factor for thyroglobulin promoter activity, strengthens the notion that the interference between transformation and the expression of the differentiated phenotype is exerted through a reprogramming of the cell transcriptional apparatus, i.e. a change in the availability of transcription factors important for the expression of cell-type-specific genes. Preliminary experiments in support of this model show that TTF-2 is also absent in transformed FRTL-5 cells (H.Francis-Lang and R.Di Lauro, unpublished observations).

Materials and methods

Solutions

Solution I: 10 mM Hepes 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF. Restore solution: 67.5% sucrose in Solution I. Solution IS: Solution I containing 6.75% sucrose. Solution II: 10 mM Hepes 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 5% glycerol. NDB: 20 mM Hepes 7.9, 0.1 M KCl, 0.2 mM EGTA, EDTA 0.2 mM, 1 mM DTT, 20% glycerol. Solution D: 20 mM Hepes pH 7.9, 0.1 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 20% glycerol.

Construction of recombinant plasmids and preparation of DNA templates

Mutant promoters were generated by site-directed mutagenesis (Kramer *et al.*, 1984). on the thyroglobulin promoter fragment extending from −168 to +36 subcloned in M13. We first constructed a pseudo-wild-type promoter (M13TgNS) by introducing two point mutations at −42 and −101 in order to generate two unique restriction sites for *Nhe*I and *Spe*I respectively. M13TgNS retains nearly 100% promoter activity (A.J.Sinclair and R.Di Lauro, unpublished). The A, B and C core mutants were constructed into the M13TgNS background by replacing the sequences boxed in Figure 5 with the sequence 5′-CAGACCTG-3′.

For the footprinting experiments shown in Figures 1 and 5 we used a restriction fragment derived from plasmid 5′-41 (Musti *et al.*, 1987) extending from −287 to +36. The footprints on the promoter mutants shown in Figure 5 were performed on restriction fragments extending from the first *Pvu*II site, located in p8CAT upstream from the promoter, to +36.

Transfection assays

The thyroglobulin promoter fragment from M13TgNS and mutations thereof were subcloned into the polylinker of the expression vector p8CAT (Riccio *et al.*, 1985). CMV-luc, a plasmid where the CMV enhancer−promoter segment (Severne *et al.*, 1988) is fused to the structural gene for luciferase was a gift of U.Deuschle. The p8CAT constructs and CMV-luc were cotransfected into FRTL-5 cells by the calcium phosphate coprecipitation technique (Graham and van der Eb, 1973). Cell extracts were prepared 48 h after transfection and CAT (Gorman *et al.*, 1982) and luciferase activities (de Wet *et al.*, 1987) were determined. The result of each transfection was calculated as: percentage chloramphenicol conversion/light units × 10 000. At least two different DNA preparations were tested for each construct.

Preparation of nuclear extracts from cultured cells

FRTL-5 and FRT cells were cultured as described (Avvedimento *et al.*, 1985). Rat-1 cells were grown in Coon's modified F-12 containing 5% calf serum. Nuclear extracts were prepared by a modification of described procedures (Dignam *et al.*, 1983; Shapiro *et al.*, 1988). Cells were scraped from tissue culture dishes in cold phosphate-buffered saline. After centrifugation, the cell pellet was resuspended in 5 vol of solution I. After 10 min on ice, the cells were collected by centrifugation, resuspended in 2 vol of solution I and homogenized in a dounce (tight pestle). Sucrose restore solution was then added (1/10 of total volume) and the homogenate was spun for 30 s at 10 000 r.p.m. in the HB4 rotor. The nuclear pellet was resuspended in solution IS, spun as above, resuspended in five pellet volumes of solution II and resuspended thoroughly by homogenization. After 30 min on ice, the nuclear suspension was spun at 35 000 r.p.m. for 30 min and 0.33 g of ammonium sulphate were added per millilitre of decanted supernatant. Proteins were allowed to precipitate for 30 min on ice, collected by centrifugation resuspended in NDB buffer and dialysed against 500 vol of NDB for 3 h. The dialysed extract was frozen in liquid nitrogen and stored in aliquots at −80°C.

Binding assays

Binding reactions for band-shift assays were assembled in solution D containing 0.2 M KCl, 2 μg poly(dI.dC) and 3 μg nuclear proteins. The reaction was started by the addition of 20−50 pg of labelled double-stranded oligonucleotide. After 15 min at room temperature, samples were loaded on a 6% acrylamide gel in Tris−glycine (Schneider *et al.*, 1986) and electrophoresed for 3 h. The gel was dried and exposed to an X-ray film with an intensifying screen at −80°C overnight. In competition experiments 50 ng of competitor were added to the binding reaction prior to the addition of labelled substrate. The DNA sequence of the oligonucleotides used are shown in Figure 1.

DNase I footprinting (Galas and Schmitz, 1978) assays were performed, on the indicated DNA fragment, according to Musti *et al.* (1987). Footprints with purified TTF-1 were performed in the presence of 100 μg/ml bovine serum albumin, 0.02% NP-40 and 0.5% polyvinylalcohol.

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