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PROTEIN BINDING DOMAINS OF THE RAT THYROGLOBULIN PROMOTER

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We have previously shown that DNA elements controlling tissue specific expression of the rat thyroglobulin gene extend 170 bp upstream of the cap site and have identified a thyroid specific nuclear factor which binds the promoter in the -60 region (site C). Here we report that the distal portion of the promoter, extending from -160 to -120, contains two contiguous DNA elements (sites A and B) which interact with the same thyroid-specific factor binding to proximal site C. A second nuclear factor, ubiquitously distributed, binds to the distal site A. Transient cotransfection-competition studies show that all the three binding sites A, B and C titrate a trans-acting factor(s) which is necessary for the transcription of the thyroglobulin gene.

The Thyroglobulin (Tg) gene is developmentally regulated, and its transcription is restricted to the thyroid gland (1). The cloned, highly differentiated rat thyroid cell line FRT-L5 (2) also exhibits specific expression of the Tg gene (3) as well as other thyroid specific functions such as iodide trapping and dependence on TSH for growth (4). In a previous study we transfected FRT-L5 cells line with scalar deletions of the 5'flanking region of the rat Tg gene (5) and determined that the minimal promoter extends 170bp from the trascriptional start site (6). The same DNA region contains the cis-elements necessary for cell-type specific transcription.

We showed that nuclear extracts (NE) prepared from FRT-L5 cells contain a factor capable of binding to the -70 region of the Tg promoter. This factor was not detectable in other cell lines which do not transcribe either the endogenous Tg gene or the transfected promoter. We have also shown that the nuclear factor could not be detected in FRT-L5 cells transformed with the v-ras oncogene (7), in which the Tg gene transcription is suppressed (8).

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However, since the minimal promoter is 170bp long, in the present study we have attempted to identify putative trans-acting factors capable of interacting, in a sequence-specific manner, with cis-acting elements of the distal part of the Tg promoter. We show that the distal part of the Tg promoter contains two protein-binding sites, A and B, interacting with factors which are necessary for the transcription of the Tg gene.

MATERIALS AND METHODS

Cell lines and DNA transfections: cell lines were grown as described (3). Test and competitor plasmid DNAs were cotransfected into the cells by the calcium phosphate precipitation technique (9). For the competition experiments the total amount of transfected plasmid DNA (50μg) was kept constant in each assay by the addition of a carrier plasmid, pUC18. Cell extracts were prepared 48h after transfections and assayed for CAT enzyme activity as previously described(10).

Plasmids: Plasmid DNAs were prepared as described (6). The constuction of 5'-17s and 5'-41 deletion mutants of the rat Tg promoter have been previously described (6). These constructs contain 170bp and 330bp respectively of the Tg 5'flanking region. Human ferritin/CAT fused gene was kindly provided by M. Marone: it contains 480 bp of human ferritin H chain 5' flanking region (11). The plasmid pPtg2 has been constructed as follows. A fragment HaeIII/AluI containing the -250 to -90 region of the Tg promoter was purified from the delition mutant 3'7, previously described (6), and cloned into the the Sma/HindIII sites of pUC18 plasmid vector.

Cloning of ds-oligonucleotides: the ds-oligos were prepared as follows. Complementary strands were synthesized on gene assembler (Beckman) and purified on a 20% PAGE 7M urea gel. The two strands were annealed, recessive ends filled with deoxinucleotides by the Klenow enzyme, and finally cloned into the Sma site of pUC18 by T4 ligase. The inserts of three recombinant plasmids, named pA40, pB13 and pC26, were analysed by nucleotide sequencing(12), they resulted to contain 3 copies of A, B and C ds-oligonucleotides respectively.

Nuclear extracts and DNAaseI footprinting: nuclear extracts were prepared as described (6). 5'end-labeled DNA fragments were obteined by T4 kinase and γ^{32}P-ATP, as described (13); 1ng of each probe (5000cpm) was incubated with proteins in a final volume of 50 ul of buffer B (50mM KCl, 25mM Tris-HCl (pH7.5), 5mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, 0.5mM PMSF,10% Glycerol) and 1μg of poly(dIdC)/20μg of NE. 100/200ng of DNAaseI was then added and allowed to digest for 2 min at 24°C.The digestion was stopped by adding equal volume of 1% SDS-20mM EDTA, followed by organic extraction and ethanol precipitation. The protection pattern was analysed on 6% sequencing gel.

Gel-retardation assays: Ds-oligonucleotides were labelled either by filling recessive ends with the Klenow enzyme and α^{32}P dNTPs, or by phoshorilating flush ends with T4 kinase and γ^{32}P-ATP. 6-8 ug of nuclear proteins were combined with 2/3μg of poly(dIdC) in a final volumes of 20ul of buffer B. Samples were incubated for 5 min at 0°C prior the addition of the probe (.5ng, 5000cpm), then incubation were continued for 10 min at 24°C. Samples were loaded onto 5% PAGE gel in 0.5x TBE buffer and electophoresed for 90' at 25mA at 4°C. Gel were dried and autoradiographed. For the competition experiments, ds-oligonucleotides were added to the reaction mixture prior the addition of the probe. Residual binding was calculated by scanning the retarded band on autoradiograms.

RESULTS AND DISCUSSION

Three protein binding sites are present in the Tg promoter: We investigated the Tg-promoter binding domains by DNAaseI footprint assay (14). The probe

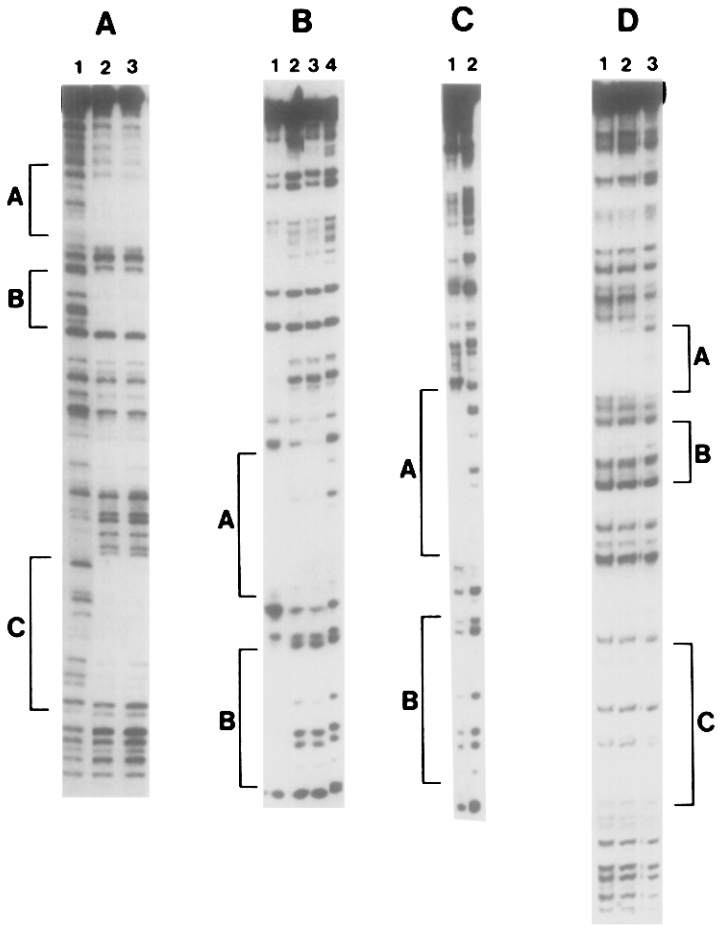
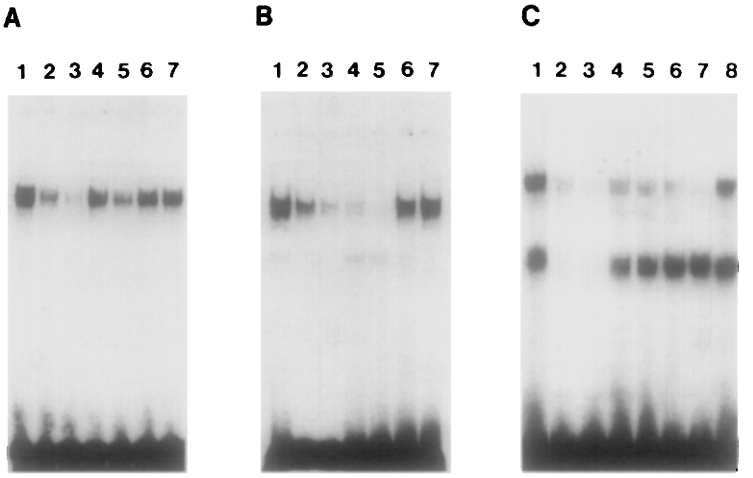


Figure 1. Protein binding sites of the rat Tg promoter. Panel A: DNAaseI footprint analysis of the BamHI/HindIII fragment of the deletion 5'17s (see methods). Lane 1: no proteins added; lanes 2 and 3: 20µg FRT-15 NE added (two indipendent extract preparetions). Panel B: Protection patterns of regions A and B generated by NE of different origin. DNAaseI footprint analysis of the EcoRI/HindIII fragment of the plasmid pPTg2 (see methods). Lane 1: 20µg of FRT-L5 NE added, lane 2: 20µg FRT NE added, lane 3:20µg BRL-3A2 (30) NE added, lane 4: no proteins added. Panel C: footprint analysis of the fragment described in panel B with 100µg of liver NE(lane 1) and in the absence of NE (lane 2).Panel D: footprint analysis of the BamHI/HindIII fragment of deletion 5'41 (see methods) with 100µg of different preparetion of liver NE (lanes 1 and 2) and in the absence of NE (lane3). Arrows indicate the extra band wich is protected with liver and BRL-3A2 NEs. Panel E: summary of footprints on the Tg promoter. Boundaries of binding sites (boxed) have been determined by electrophoretic fractionation of DNAaseI digestion run in parallel with Maxam-Gilbert sequencing (20) reactions of the same fragment (data not shown). The underlined sequences represent the common motif present in all three binding sites. Arrows indicate the palindrome included in the binding site C.

used was a DNA fragment, from +39 to -167 relative to the transcriptional start site, which contains the minimal Tg promoter sequences. Fig.1A shows the DNAaseI protection pattern obtained with this probe after incubation with nuclear extract (NE) obtained from FRT-L5 cells. Three protected regions (indicated as A, B and C) are observed. The precise boundaries of the protected regions, which we identify as protein binding sites, are shown in Fig.1E. Site A maps between position -156 and -141, site B from position -133 to -120, relative to the cap site. Site C span from position -76 to -61. Since



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A-38MER	-164	TACTGATTACTCAAGTATTCTTAGCGGGAGCA AATGAGTTGATAAGAATCGCCCTGGTCTGAGT	-127
B-21MER	-138	GGAGCAGACTCAAGTAGAGGG CCTCGTCTGAGTTCATCTCCC	-117
C-22MER	-78	GCCCAGTCAAGTGTGTTCTTGAAC CGGGTCAGTTCACACAAGAACTTG	-58

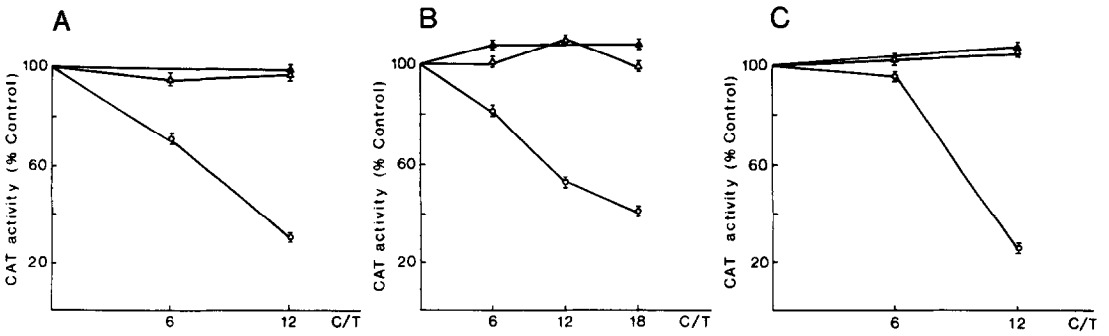
Figure 2: Gel retardation analysis of single binding sites A, B and C. Panel A: end-labeled ds-oligonucleotide C-22mer, containing site C, was incubated with FRT-L5 NE (8µg) in the absence or in the presence of various quantities of unlebeled ds-oligonucleotides. Lane 1: no competitor; lanes 2 and 3: 100 and 250 fold excess respectively of cold homologous C-22mer; lanes 4 and 5: 100 and 250 fold excess respectively of cold B-21mer; lanes 6 and 7: 250 fold excess of a cold 22-mer oligonucleotides, conteaining the "CAAT" region of the human C-reactive protein gene (G.Ciliberto, personal comunication) (lane 6) and a 60-mer oligonucleotides containing the "repressor" binding region of the rat retinol-binding gene (lane 7) (21). Panel B: end-labeled ds-oligonucleotide B-21mer, containing site B, was used in gel-retardation assay as described for panel A. Lane 1: no competitor; lane 2 and 3: 100 and 250 fold excess respectively of cold B-21mer; lane 4 and 5: 100 and 250 fold excess respectively of cold C-22Mer; lane 6 and 7, as described for the corresponding lanes of panel A. Panel C: end-labeled ds-oligonucleotide A-38mer, containing site A, was incubated with FRT-L5 NE (8µg) in the absence or in the presence of various oligonucleotides. Lane 1: no competitor; lanes 2 and 3: 100 and 250 fold excess respectively of cold A-38mer; lanes 4 and 4: 100 and 250 fold excess respectively of cold B-21mer; lanes 6 and 7: 100 and 250 fold excess respectively of cold C-22mer; lanes 8: as described for lanes 7 of panels A and B. Panel D: Nucleotide sequence and ds-structure of synthetic oligonucleotides.

we have previously shown that site C binds to a thyroid-cell-specific factor (6) the question was wether the nuclear factors interacting with sites A and B were restricted in the thyroid cells. Fig.1B shows DNAseI protection patterns of a DNA fragment containing the -250 to -90 region of the Tg promoter after incubation with comparable amounts of NEs of several cell types. Fig.1B shows that the FRT-L5 NE (lane 1) generates two major footprints corresponding to sites A and B. The additional protection which is detectable upstream site A was not reproducible in several indipendent experiments. Nuclear extracts from different cell-lines (FRT(15) lane 2 and BRL(16) lane 3) not expressing Tg gene, appear to protect only site A. A 10 fold excess of liver NE does not change the protection-pattern (Fig.2C). Incubation of liver NE with a probe bearing also site C does not show protection of this site (Fig.2D). In summary these data indicate that the 170bp-long promoter region of the Tg gene contains three sites A, B, C, which bind nuclear factors of thyroid-cells. Site B and C bind a factor which is present only in nuclear extracts originating from cells expressing the Tg gene, while site A binds a factor(s) present in several cell-types. It is noteworthy that nuclear extract from liver tissue and from BRL-3A2 cells generate a more extended protection at the 5' boundary of site A (see arrows in Fig.1B and 1C). This may reflect different binding affinities, for this particular region, of components of NE of different cells.

The same thyroid trans-acting factor binds to the three independent sites of the Tg promoter: Inspection of the nucleotides sequences of the three binding sites A, B and C reveals that a common motif, TCAACT, is present in all three sites and an inverted copy, PyCTTGA, is present at the 3' boundary of site C (see Fig. 1E). We have tested, by cross-competition experiment in"gel retardation assay"(17), whether sites B and C bind to the same thyroid specific factor that we identify as TgTF1. Two ds-oligoligonucleotides, the B-21mer and the C-22mer (Fig.2D), homologous to sites B and C respectively, were end-labeled (see methods) and incubated with the nuclear extract of FRT-L5 cells. Fig.2A shows that TgTF1 binding to a "hot" C-22mer is largely competed by the same unlabeled oligonucleotide but only partially by the B-21mer. The reverse experiment (Fig.2B), in which a "hot" B-21mer is used, reveals that the thyroid factor binding to site B could be equally competed by both the cold B-21mer and the cold C-22mer. Comparison of the amount of "cold" oligonucleotide required to achieve 50% competition reveals that the C-22mer competes for site B twice as efficiently as the B-21mer itself. These data suggest that the same thyroid factor TgTF1 binds to sites B and C of the promoter and that the proximal site C is of higher intrinsic affinity than the distal site B. The sequence homology of the three sites suggests that also site A should bind TgTF1, besides the ubiquitous factor. Gel retardation

assays (Fig.2C) of a ds-oligonucleotides, A-38mer (see Fig.2D) corresponding to site A, showed the formation of two sequence specific complexes of different sizes between site A and FRT-L5 NE; only one complex (the slower-migrating one) was competed by an excess of cold oligonucleotides containing the sequences of either site B or site C. These data suggest that site A contains DNA sequences interacting with at least two different factors. We propose that TgTFI interacts with the three sites described above (A, B and C), in addition an ubuiquitous factor binds site A.

The three binding sites A, B, C interact with trans-acting factor required for Tg transcription: The transcriptional relevance of the thyroid specific factor TgTFI was investigated in a series of transient-cotrasfection competition studies. Three oligonucleotides , A-38mer, a B-42mer and a C-54mer (Fig.3D), containing the binding sites A B and C respectively, were tested in their ability to decrease the expression of the bacterial CAT gene fused to the Tg promoter. Each ds-oligonucleotide was cloned in the vector plasmid pUC18 (see method) and used as competitor DNA in "in vivo" assays. An optimal subsaturating amount of DNA (50ug), containing the entire Tg promoter fused to the bacterial CAT gene, was cotransfected with increasing amounts of each competitor DNA into FRT-L5 cells. As shown in Fig.3 each of the three



D

A-38MER -164 TACTGATTACTCAAGTATTCTTAGCGGGAGCA
AATGAGTTGATAAGAATCGCCCTGGTCTGAGT -127

B-42MER -134 CAGACTCAAGTAGAGGGAGTTCCTGTGACTAGCAG
GTTCATCTCCCTCAAGGACACTGATCGTCTCTTTTG -93

C-53MER -98 GAAAACAAAAGTGAGCCACTGCCCAGTCAAGTGTTCTTGAACAGTAGAGC
GTTTTCACTCGGTGACGGGTCAGTTCACAAGAACTTGTCATCTCGTGACG -46

Figure 3: In"vivo" competition experiments. 50ug of 5'-17s (10), cicken βactin (18) or h-H ferritin (11) chimeric genes and variable amounts of the competitor were cotrasfected as calcium phosphate precipitate in subconfluent FRTL-5 cells. The levels of CAT activity were plotted as % of uncompeted reaction against the molar ratio of competitor:test gene. The competitors used (see Materials and Methods) are :(panel A) pA40 representing site A;(panel B) pB13 representing site B;(panel C) pC26 representing site C. The experimental points in the figure are the means ± SD determined for each reaction in several independent trasfection experiments. Panel D: Nucleotide sequences and ds-structures of oligonucleotides which have been cloned (see methods).

competitors reduces the expression of the Tg-promoter/CAT gene construct. As controls we have tested two different chimeric genes, the chicken β actin-promoter-CAT (18) and the human H-Ferritin-promoter-CAT (see methods) constructs. When these constructs were cotransfected with each of the three described competitors, CAT activities did not decrease. These data suggest that the thyroid specific factor TgTFI is present in the FRT-L5 cell line in limiting amounts and it is required to promote transcription of the Tg promoter.

In conclusion we have shown that the promoter region of the rat thyroglobulin gene has multiple DNA binding domains which seem to be involved in the regulation of transcription. These cis-acting promoter elements seem to to serve as binding sites for a thyroid specific factor, TgTFI, which binds to each site with different affinities. A second factor, ubiquitously distributed, binds exclusivly to the distal site A. It is likely, as suggested inproposed models (19), that interactions between molecules of TgTFI, as well as interactions between this factor and the ubiquitous one, are required for the optimal stimulation of Tg promoter transcription.

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REFERENCES

1. Di Lauro, R., Obici, S., Acquaviva, A.M., and Alvino, C. G. 1982. Gene 19:117-125.
2. Ambesi-Impiombato, F.S., Parks, L. A.M., and Coon, H.G. 1980. Proc. Natl. Acad. Sci. U.S.A. 7:3455-3459.
3. Avvedimento, V.E., Monticelli, A., Tramontano, D., Polistina, C., Nitsch, L., and Di Lauro, R. 1985. Eur. J. Biochem. 149:467-472.
4. Avvedimento, V.E., Tramontano, D., Ursini, M.V., Monticelli, A., and Di Lauro, R. 1984. Biochem. Biophys. Res. Commun. 122:472-477.
5. Musti, A.M., Avvedimento, V.E., Polistina, C., Ursini, M.V., Obici, S., Nitsch, L., Cocozza, S., and Di Lauro, R. 1986. Proc. Natl. Acad. Sci. U.S.A. 83:323-327.
6. Musti, A.M., Ursini, M.V., Avvedimento, V.E., Zimarino, V., and Di Lauro, R. 1987. Nucl. Acids Res. 15:8149-8166.
7. Avvedimento, V.E., Musti, A.M., Fusco, A., Bonapace, M.M, and Di Lauro, R. 1988. Proc. Natl. Acad. Sci. U.S.A. 85:1744-1749.
8. Colletta, G., Pinto A., Di Fiore, P.P., Fusco, A., Ferrentino, M., Avvedimento, V.E., Vecchio, G., and Tschuda, N. 1983. Mol. Cell. Biol. 3:2099-2109.
9. Graham, F.L. and Van der Eb, A.J. 1973. Virology 52:456-467.
10. Gorman, C.M., Moffat, L.F. and Howard, B.H. 1982. Mol. Cell. Biol. 2:1044-1051.
11. Santoro, C., Morone, M., Ferrone, M. Costanzo, F., Colombo, F., Minyanti, C. Cortese, R. and Silengi, L. 1986. Nucl. Acidis Res. 14:2863-2876.

12. Sanger, F., Nicklen, S. and Coulson, A;R. 1977. Proc. Natl. Acad. Sci. USA.74:5463-5467.
13. Maniatis, T., Fritsh, E.F. and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual(Cold Spring Harbor Laboratory,Cold Spring Harbor, N.Y.).
14. Galas, D., and Schimtz, A. 1978. Nucl. Acids Res. 5:3157-3170.
15. Nitsh, L., Tramontano, D., Ambesi-Impiombato, F.S., Quarto,N., Bonatti, F. 1985. Eur.J.Biol. 38:57-66.
16. Nissley, S.P., Short, P;A., Rechler, M.M., Podskalny, J.M. and Coon, H.G. 1977. Cell 11:441-446.
17. Garner, M. M., and Revzin, A. 1981. Nucl. Acids Res. 9:3047-3060.
18. Billeter, R., Quitschke, W and Paterson, B. 1988. Mol. Cell. Biol. 8:1361-1365.
19. Ptashne, M. 1988. Nature 335:683-689.
20. Maxam, A.M. and Gilbert, W. 1980. Meth. Enzymol. 65:499-560.
21. Colantuoni, V., Pirozzi, A., Blance, C. and Cortese, R. 1987. Embo J. 3:631-636.