CLONING AND CHARACTERIZATION OF THE HUMAN THYROID HORMONE RECEPTOR β1 GENE PROMOTER

Akihiro Sakurai*, Takahide Miyamoto and Leslie J. DeGroot

Thyroid Study Unit, The University of Chicago, Chicago, IL 60637

Received April 2, 1992

SUMMARY: The promoter region of the human (h) thyroid hormone receptor (TR) β1 gene was isolated from a human placenta genomic library. Primer extension and S1 nuclease mapping confirmed a single transcriptional start site. DNA sequence analysis of the 5' upstream region revealed the existence of a putative thyroid response element (TRE) which is highly homologous to TREs found in several thyroid hormone responsive genes. Binding of hTR protein to the promoter region of the hTRβ1 gene was confirmed by gel mobility shift assay. A transient transfection study demonstrated that hTR activated the expression of a reporter gene containing the promoter sequence of the hTRβ1 gene in a hormone dependent manner.The TRE in the hTRβ1 gene promoter may be involved in the autoregulation of hTRβ1 gene expression.

Thyroid hormone plays important roles in growth, development, and metabolism of vertebrates. These effects are mediated via specific thyroid hormone receptors (TRs) which are cellular homologues of v-*erb*A. The existence of two genes encoding TRs (α and β) and their subtypes is known (1,2).

The general mechanism by which TRs regulate the expression of T_3 responsive genes involves the binding of TRs to thyroid hormone response elements (TREs) in those genes. TRE sequences in natural genes are usually found as a unit consisting of several imperfect elements (3).

Expression of several members of the *erb*A-related nuclear receptor superfamily (receptors for retinoic acid, glucocorticoid, progesterone, estrogen and vitamin D) is regulated by treatment with their cognate ligands. Genes encoding retinoic acid β receptor and progesterone receptor contain protein binding sites (hormone response elements) for their own translation products in their promoter regions (4,5). Receptor proteins bind to those elements and control their own expression. TR mRNA levels

---

*Present Address: Department of Geriatrics, Endocrinology and Metabolism, Shinshu University School of Medicine, Matsumoto, 390 Japan

0006-291X/92 $4.00

are also reported to be up- or down-regulated by thyroid hormone in a tissue specific manner (6). Rat TRβ1 is up-regulated by T3 in pituitary. These observations imply the possible existence of auto-regulation of TR expression. To better understand the mechanism of hTRβ1 expression and its regulation, we isolated and characterized the promoter region of the hTRβ1 gene.

Materials and Methods

Screening of the library: A human placenta genomic DNA library was screened using a nick translated hTRβ1 cDNA probe (pheA4) (7). Positive clones were restriction enzyme-mapped followed by subcloning into M13 vector, and sequenced by the dideoxy chain termination method (8).

Primer extension: A ^{32}P-end labeled primer (+166~+147, +1 at transcription start site of hTRβ1 cDNA) was hybridized to 20 μg of human brain RNA, and single stranded cDNA was synthesized in the presence of 0.5 mM of each dNTP using avian myeloblast virus reverse transcriptase.The product was ethanol precipitated and resolved on an 5% sequencing gel along with M13 template.

S1 nuclease mapping: A ^{32}P-end labeled synthetic primer (+49~-11 of hTRβ1 cDNA) was incubated with 10 μg human brain RNA overnight at 50°c. Three hundred units of S1 nuclease were added and incubation was continued at 37°c for 1 h. Following precipitation, the product was dissolved in sequence gel loading buffer and resolved on an 5% sequencing gel.

Gel mobility shift assay: 30,000 cpm of ^{32}P end-labeled oligonucleotide containing -189~-159 of hTRβ1 (β1TRE) were incubated with nuclear extracts from COS-7 cells transfected with expression vectors for hTRα1 or hTRβ1 (9). Protein-DNA complexes were separated from protein-free DNA by non-denaturing gel electrophoresis in 5% polyacrylamide gels. Gels were run at a constant voltage of 200 V for 120 min, and exposed to Kodak XAR-5 film. [^{35}S]Methionine-labeled *in vitro* translated hTRα1 and hTRβ1 were also incubated with either the *cis*-acting element of Adenovirus 5 (10) or β1TRE and applied to electrophoresis as described above.

CAT assay: β1TRE was subcloned into pUTKAT1 (11). This reporter plasmid (4μg) with or without hTR-expression vector (1μg)(9) was transfected into COS-7 cells by the $CaPO_4$ coprecipitation method. After incubation for 48 h in the presence or absence of 5nM T3 in the culture medium, cells were harvested and CAT activity was measured in cell extracts(12). CAT activation by host vector (pCDM8)(13) in the absence of T3 was normalized to 1.0.

Results and Discussion

Screening of a human placenta genomic library identified two positive clones (λPG26 and λPG25, Fig. 1). These clones were restriction enzyme-mapped and fragments containing exons and adjacent introns were sequenced by the dideoxy chain termination method. λPG26 contained 171 bp of the 5' end sequence of the reported hTRβ1 cDNA (7) and a typical "TATA" box, but lacked a "CAAT" box. λPG26 also contained five GGGCGG motifs (Fig. 2).

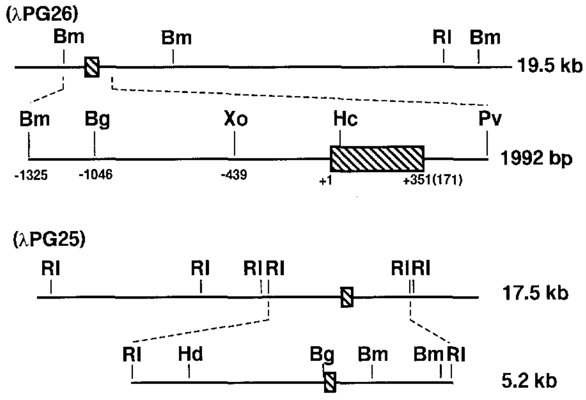

Figure 1. Physical map of two genomic clones, λPG26 and λPG25. Exons are shown as hatched boxes. Positions of several restriction sites are also shown. Bg; *Bgl* II, Bm; *Bam* HI, Hc; *Hinc* II, Hd; *Hind* III, Pv; *Pvu* II, RI; *Eco* RI, Xo; *Xho* I.

To determine the transcription start site(s) of hTRβ1 gene, primer extension and S1 nuclease mapping were carried out (Fig. 3). For primer extension, a ^{32}P end-labeled synthetic oligonucleotide complimentary to the sequence 173 ~ 192 bp downstream of the putative TATA box was hybridized to human brain total RNA followed by extension with reverse transcriptase. The primer extension products terminated at two consecutive nucleotides, G and T (Fig. 3B). This area was further examined by S1 nuclease mapping. A ^{32}P end-labeled 60 bp oligonucleotide containing these terminating nucleotides was hybridized to human brain total RNA and digested with S1 nuclease. S1 digestion produced a single band of 49 bp (Fig. 3C). From these results, the G residue 180 bp upstream of the 5' end of the reported hTRβ1 cDNA sequence (7) was determined to be a unique transcription start site. Therefore, lPG26 contains the first exon of the hTRβ1 gene, which is 351 bp in length. Sequence analysis revealed that λPG25 contains the second exon (nucleotide 172 ~ 243 according to numbering by Weinberger *et al.*,) (7) (Fig. 1, sequence not shown). These results indicate that the 5' non-coding region of hTRβ1 cDNA is split into at least three exons as predicted from the results of genomic Southern analysis (14).

TRs bind to TRE sequences of thyroid hormone responsive genes and regulate the expression of those genes. The consensus sequence of TRE has been proposed by Brent *et al.* to be AGGT(C/A)A (3). Nucleotides -185 ~ -161 of the hTRβ1 gene promoter have homology to TREs found in other thyroid hormone responsive genes, and to the consensus TRE sequence (Fig. 2). To examine whether this region is a binding site for TRs, an oligonucleotide containing these sequences (β1TRE) was synthesized and used for gel mobility shift assay. Double retarded bands were observed when nuclear extracts from COS-7 cells transfected with hTRα1 or hTRβ1 expression vector were incubated with ^{32}P end-labeled oligonucleotide (Fig. 4A). These retarded bands were sequence specific since a 50-fold molar excess of unlabeled oligonucleotide containing a palindromic TRE sequence (15) displaced

```
...-1325                                                        ggatc -1321
...-1320    catatcgtcataactctattatactgcgtgttacacgaatagatgtgaatattaaattat -1261
...-1260    gatgtcggaattattttaatactgtctataagaaattaattgtactctgttgtcaaagta -1201
...-1200    gttttgttgcaactatagttccttattgactaccttttagctgagtgaggactcggtatt -1141
...-1140    tcccaagtatccttctagctcagaaagcaagtctcttcctggtctccaggacttaaggtc -1081
...-1080    gggggcatttgagagacctatatttgcccgggaaagatctcttgaagagtatacattatt -1021
...-1020    tttgtcttcctggttttatctatacatttccagggcaaagacaaattaatagggcggccg -961
....-960    ttctgggacctgaggagtgtctcagccctgtacgcgcctctcccacgatatgcataatgg -901
....-900    cggtggggcggggtgtcctccttaagggcaacccaggcacgtcccaaacttccattcg -841
....-840    tggggtggccgtggtggttaagagagctgccagatggtcggaccagcggaggccccaaga -781
....-780    agagccagagcgccctgtattcccgacacgcgccacaagtggtttaggagcggagggagg -721
....-720    agcgctgcgcacgggtcggctcggcgggcaaagaaggcgagacgtgctcctggaagactc -661
....-660    gccctccgtcccgtgtcactcgttccccattctttcctcttctccaactagagtaatgac -601
....-600    gcccaaacccgcacctatgcgcgcaggcaatcgggtgggtggacgcgcggccaccaaacc -541
....-540    cacagcaccgtcaccaaccctgggagggcacggccgggattaggaggagggggagcgccc -481
....-480    acacctggggaggcaggtgcggaggcggccgccccggggacctcgagtgcgtaggactcg -421
....-420    gggtcggggtcggggtcggcaagccggcgctgtgagcgcgtcggagcgctggcccggagc -361
....-360    atggcagggggcgtctgtaccggctgggcagcccggggcggtggcgatggcggtggcggc -301
....-300    ggcggcggggtgtgcggccaggaggcatttcctcgctgcgcccctgggcagcgggtttgc -241
....-240    ctgctcttggccgccgccgccaccgccgcgcaaagtcggacagccgtgaggctggaggga -181
....-180    aaccaggtcaccggttgccaagagccggcgcggagcaaggcgcccgggcccggagtaaga -121
....-120    cagcgcccgggaagcgggccggggcggggccgggcacgcgggggacccggagaggcgggga -61
.....-60    ctctggtgccccagccgcagtagcttcctacgcctataaaagtggagagaccggggaggt -1

.......1    GCGGCCGGCCCTGGCTGCGGCCGCCTCTCTTCGCCCAAGGAGTTGACATTTTGCAGGACT  60
......61    CGCGCGACGCCCAGTCGCCGGCGCTCCCCGGGACCCCGCCGCCGGAGGAGGGGGCGGAGG  120
.....121    AGGGTGGAGACTGCGGGGCTTGGCCAAGGAAGGCGCACATCCTCGGGCGGGCGGCGTGAC  180
...181(1)   CGGCGGGGATTAACTTTGCATGAATAATGTGAGTGCGCTTGGAAAAGAGACCTCCTGCTC  240(60)
..241(61)   CGCGGGCTCGGGGCAAGAGCCCGCAGGCTACCTTCCCCGGGCAGGGGCGCTCAACCCAAC  300(120)
.301(121)   CGGCTCCAGGGCACTGGTAATTTGGCTAGAGGACCGCGCGGAGGCAGCGGG           351(171)

........    gtaagaggaggagggggcgacagttccaactgtccacagggtgggcgggatggtgacgga
........    gcgtcgcaagaaccggaggggtgcgggcggctaagccgagcgcgcgcgggcgggcaggcg
........    ggtgagcgtgggtggtgggggtgtcatcagcctgattacctgcctccggcgcttctgcgc
........    cccggatctgggaggaccgctctcgtgttcgggcgaccgcggcggcaggctgggagctac
........    ggagtggacagtggtggaacagggtggccgggctctcgttccaatgcagcggctctgttc
........    ctcaaaccccaagccagctg...;................................
```

Figure 2. Nucleotide sequence of a 1992 bp fragment of λPG26. Capital letters and lower case letters indicate exonic and intronic sequence, respectively. The transcription start site is numbered +1 (See Fig. 3). Nucleotide numbering by Weinberger *et al.* (7) is shown in parentheses. The TATA box sequence is underlined and recognition sites for the Sp1 transcription factor are boxed. Putative thyroid response elements are indicated by arrows.

this binding (lanes 3 and 6), while the same amount of irrelevant DNA having the sequence of the *cis*-acting element of Adenovirus 5 showed no effect (lanes 4 and 7). When [^{35}S]methionine-labeled *in vitro* translated hTRα1 or hTRβ1 was incubated with β1TRE, a single retarded band was observed (Fig. 4B, lanes 2,4). On the other hand, no retarded bands were seen when the *cis*-acting element of Adenovirus 5 was used

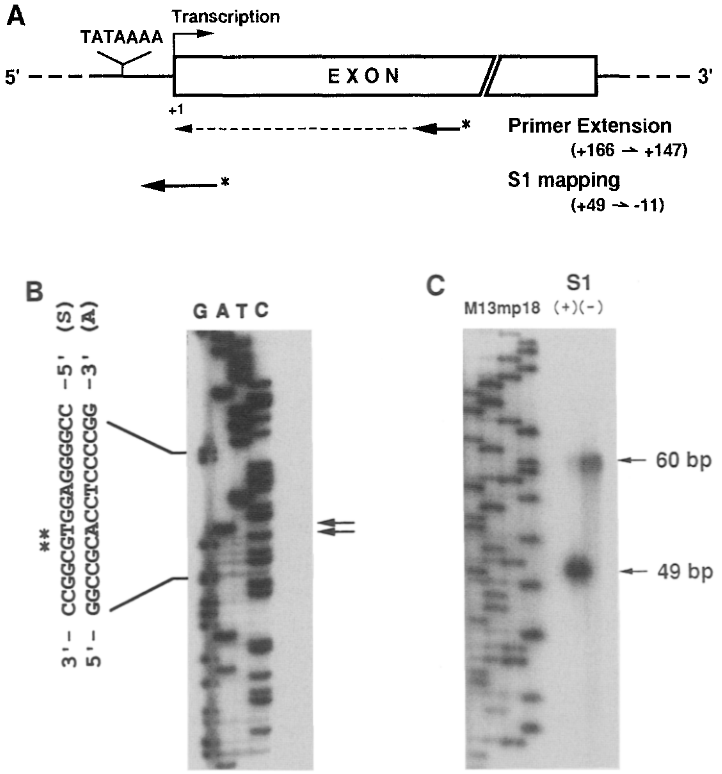

Figure 3. **A)** Schematic representation of the protocol for primer extension and S1 nuclease mapping. Arrows indicate oligonucleotides used for each experiments. **B)** Primer extension. M13 template containing a fragment of λPG26 was sequenced using the same oligonucleotide used for primer extension, and electrophoresed along with the primer extension product. The position of termination products are indicated by arrows, and corresponding nucleotides in DNA are indicated by asterisks.(S) and (A) are sense- and antisense-strand sequence, respectively. **C)** S1 nuclease mapping. Synthetic oligonucleotide was incubated with (+) or without (-) S1 nuclease after hybridization to human brain RNA, and resolved on a sequencing gel along with the sequenced M13 mp18 vector.

(lanes 1,3). The reason why COS-7-expressed hTRs and *in vitro* translated hTRs produce a different number of retarded bands is not clear, but may be due to the lower concentration of the *in vitro* translation products. The additional bands generated by COS-7 nuclear extracts may also represent the binding of TR-auxiliary protein (TRAP) (16) to β1TRE. TRAP is reported to bind to a pentanucleotide consensus sequence (T/A)GGGA (17) and β1TRE contains an AGGGA motif at -185 ~ -181 (Fig. 2).

The transcriptional activity of β1TRE was estimated by a transient expression study. As shown in Fig. 5, transcription of a reporter plasmid containing β1TRE was activated in a T3 dependent manner when expression vectors for hTRα1 or hTRβ1 were cotransfected. On the other hand, cotransfection of the host expression vector

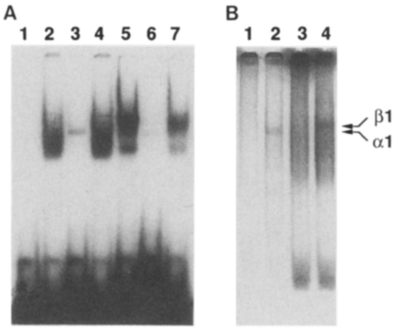

Figure 4. **A)** β1TRE was ^{32}P end-labeled and incubated with COS-7 nuclear extracts transfected with pCDM8 (lane 1), expression vector for hTRα1 (lanes 2-4) or hTRβ1 (lanes 5-7) followed by gel mobility shift assay. A 50-fold molar excess of palindromic TRE was added for lanes 3 and 6, and control DNA from the *cis*-acting element of Adenovirus 5 was added for lanes 4 and 7. **B)** [^{35}S]Methionine-labeled *in vitro* translated hTRα1 (lanes 1,2) or hTRβ1 (lanes 3,4) were incubated with either the *cis*-acting element of Adenovirus 5 (lanes1,3) or β1TRE (lanes 2,4), and applied to the gel mobility shift assay. Retarded bands seen in lanes 2 and 4 are indicated by arrows.

(pCDM8) or an expression vector for the non-hormone binding TR variant, hTRvα2, had no effect.

From these results we conclude that the hTRβ1 gene promoter contains a TRE sequence which could activate the transcription of hTRβ1 in a hormone dependent manner. Isolation of the hTRβ1 gene promoter region will be helpful for studying regulation of hTRβ1 expression. *In vivo*, rat TRβ1 mRNA level is up-regulated by T3 in the pituitary while it is unaffected in other tissues examined (6). Although the basal expression of hTRβ1 is not T3 dependent, and it is not known if up- regulation by T3 occurs at the transcriptional level, our results imply that TRs may be involved in and necessary for T3 dependent up-regulation of TRβ1 mRNA expression. The coregulation by some pituitary specific factors along with TRs seems likely. Alternatively, other factors which negatively regulate the TRβ1 expression might exist

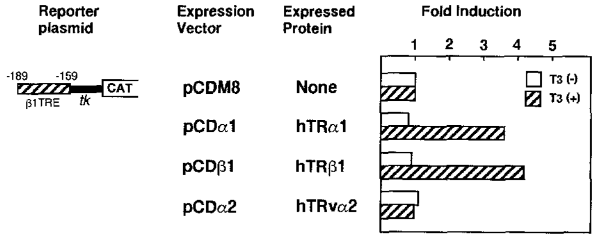

Figure 5. T3 dependent transcriptional activation by various hTRs of a reporter plasmid containing the β1TRE. CAT activation by pCDM in the absence of T3 was normalized to 1.0. Each bar represents the mean value of three independent determinations.

in tissues other than the pituitary. We are currently investigating the hormone dependent transcriptional regulation of hTRβ1 gene by hTRs and other nuclear receptors using promoterless reporter constructs with various deletions.

Acknowledgments

The authors wish to thank Drs. C. Weinberger and R. M. Evans for providing pheA4, Drs. A. Aruffo and B. Seed for providing pCDM8, and Dr. D. D. Moore for providing pUTKAT1. This work was supported in part by USPHS Grants DK-13377, the March of Dimes Birth Defects Foundation, and the David Wiener Research Fund.

References

1. DeGroot,L.J., Nakai,A., Sakurai,A. and Macchia,E. (1989) *J. Endocrinol. Invest.* **12**, 843-861.
2. Lazar,M.A. and Chin,W.W. (1990) *J. Clin. Invest.* **86**, 1777-1782.
3. Brent,G.A., Harney,J.W., Chen,Y., Warne,R.L., Moore,D.D. and Larsen,P.R. (1989) *Mol. Endocrinol.* **3**, 1996-2004.
4. Sucov,H.M., Murakami,K.K. and Evans,R.M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5392-5396.
5. Turcotte,B., Meywe,M-E., Bellard,M., Dretzen,G., Gronemeyer,H. and Chambon,P. *J. Biol. Chem.* **266**, 2582-2589.
6. Hodin,R.A., Lazar,M.A. and Chin,W.W. (1990) *J. Clin. Invest.* **85**, 101-105.
7. Weinberger,C., Thompson,C.C., Ong,E.S., Lebo,R., Gruol,D.J. and Evans,R.M. (1986) *Nature* **324**, 641-646.
8. Sanger,F., Coulson,A.R., Barrell,B.G., Smith,A.J.H. and Roe,B.A. (1980) *J. Mol. Biol.* **143**, 161-178.
9. Nakai,A., Sakurai,A., Macchia,E., Fang,V. and DeGroot,L.J. (1989) *Mol. Cell. Endocrinol.* **72**, 143-148.
10. Hearing,P. and Shenk,T. (1983) *Cell* **33**, 695-703.
11. Prost,E. and Moore,D.D. (1986) *Gene* **45**, 107-111.
12. Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.* **2**, 1044-1051.
13. Aruffo,A. and Seed,B. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8573-8577.
14. Sakurai,A., Nakai,A. and DeGroot,L.J. (1989) *Mol. Cell. Endocrinol.* **71**, 83-91.
15. Glass,C.K., Holloway,J.M., Devary,O.V. and Rosenfeld,M.G. (1988) *Cell* **54**, 313-323.
16. Burnside,J., Darling,D.S. and Chin,W.W. (1990) *J. Biol. Chem.* **265**, 2500-2504.
17. Beebe,J.S., Darling,D.S. and Chin,W.W. (1991) *Mol. Endocrinol.* **5**, 85-93.