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THE THYROID HORMONE INHIBITS THE THYROTROPIN RECEPTOR PROMOTER ACTIVITY: EVIDENCE FOR A SHORT LOOP REGULATION

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Thyrotropin, by binding its specific receptor on the plasma membrane of the thyrocyte, regulates thyroid function and differentiation. In FRTL5 cells, thyrotropin down-regulates the thyrotropin receptor (TSHr) promoter activity and induces the transcription of the alpha form of thyroid hormone receptor (TR-α1). In this study we show that the thyrotropin receptor down-regulation, induced by thyrotropin, is mediated by TR-α1. The thyroid hormone receptor binds, *in vitro*, the thyrotropin receptor minimal promoter and inhibits promoter activity in cotransfections experiments in CV 1 cells. The inhibition is achieved only in the presence of the thyroid hormone. The TSHr promoter mutated in the thyroid hormone receptor binding site does not bind TR, *in vitro*, and its activity is not inhibited, in cotransfection experiments, in CV 1 cells. The same mutation abolishes the TSH mediated down-regulation of the TSHr promoter activity in FRTL5 thyroid cells. These results support the hypothesis of a regulatory short loop of thyroid hormone in thyroid cells.

The thyrotropin receptor (TSHr) is a thyroid-specific protein and the binding of thyrotropin (TSH) to its receptor is a critical event in the control of thyroid function and differentiation (1,2,3). TSH exerts its biological effects mainly activating adenylate cyclase but also the phosphatidyl inositol pathways (2). TSH is known to

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positively control general protein synthesis as well as tissue-specific gene expression in thyroid (1). Recently it has been shown that in FRTL5, a differentiated rat thyroid cell line, TSH negatively regulates the gene expression of its own receptor (4). The addition of TSH to FRTL5 cells causes a down-regulation of TSHr gene transcription, and conversely the withdrawal of the hormone from the culture medium activates the transcription of this gene. The TSHr minimal promoter has been identified within -199 and -39 base pairs in the 5' flanking region upstream of the ATG initiation codon. The minimal promoter lacks a TATA-box and like other TATA-less housekeeping gene promoters, has a high G+C content. However, in transfection assays, it is preferentially expressed in thyroid cells and it is negatively regulated by TSH or forskolin (5). A *cis* DNA element similar to cAMP response element (CRE-like) has been identified in the minimal promoter, between -149 and -127 base pairs, and it has been shown that this element works as a constitutive enhancer of TSHr promoter activity (6). We have shown, in a previous paper, that TTF1, a thyroid specific transcription factor, binds to the minimal TSHr promoter and is able to transactivate it in a cotransfection assay (7). Hence TTF1 participates, at least partially, to restrict the activity of the TSHr promoter to thyroid cells .

Thyroid hormones (T4/T3), the major product of differentiated thyroid cells, affects a very large number of biological processes. It is involved in development, tissue differentiation and cell growth (8). T3 functions via binding to a specific nuclear thyroid receptor (TR) and the resulting complex binds the thyroid hormone responsive element (TRE), a *cis* acting element found in promoters of T3 responsive genes (9, 10). There are at least two forms of TR, $\alpha 1$ and $\beta 1$, encoded by two different genes (9). TR binds to DNA via its zinc-finger domain and can bind the TRE as a monomer, homodimer and heterodimer with the thyroid receptor auxiliary proteins, TRAPS (11). The leucine zipper domain of the TR is required for the protein-protein interaction with the TRAPS. The best characterized TRAP is the retinoic X receptor (RXR) (11) and the heterodimerization with RXR is required for efficent, *in vitro*, TR binding to TRE (9, 10, 11). The consensus binding sequence of TRE is A/GGGTC/AA (half site) typically arranged as two or three elements forming palindromes, direct or inverted repeats but also as a single half-site (9). The TR has the dual ability to activate or repress transcription of the responsive genes (10). The negative TRE (nTRE) is less clearly defined than the positive TREs. nTREs have been identified in the TSH-β subunit gene promoter (12, 13).

Although papers published more than 40 years ago have reported that T3/T4 acted directly on the thyroid gland to inhibit its response to TSH (14), little is known, at the molecular level, about the activity of the thyroid hormone on the thyroid cells. An inhibitory effect of T4/T3 on the activation of adenyl cyclase was described in

human thyroid tissue (15). Iodothyronine 5'-deiodinase (5'-D) gene expression is regulated positively in FRTL5 cells both by TSH and T3 (16). In the same cell line both TR forms, α1 and β1 can be found. The β1 messenger RNA was detected in FRTL5 grown with and without TSH but the α1 form mRNA was detected only in presence of the hormone (17).

In this study, we investigated the role of the complex T3/TR on the TSHr promoter activity. We show that in the minimal TSHr promoter there is a TRE sequence, 5'TGAGGTCA3', corresponding to the half site of TR recognition element (18) and that TR binds to this site in vitro. We further demonstrate that in transfection experiment in CV-1 cells, the TSHr promoter is down-regulated by T3 when it is complexed with the TRα1. We have constructed a TSHr promoter mutant that does not bind the TR-α1 in vitro and is not inhibited by the complex T3/TR-α1 in CV 1 cells. The mutagenized promoter activity is not down-regulated by the presence of TSH in FRTL5 cells.

MATERIALS AND METHODS

Cell culture and transfection

CV 1 cells were grown and transfected by the calcium phosphate protocol according to Pfahl et al. (19). The depletion of thyroid hormone in serum used in the transfection experiments was performed according to Samuels et al. (20). The cells, grown in 10-mm tissue culture dishes, were incubated overnight with a calcium phosphate precipitate containing 3 μg of the reporter plasmid, pGE-T200 or a mutated TSHr promoter, 3 μg thyroid receptor α1 expression vector [kindly provided by Dr. A. Farsetti, Ist. Regina Elena, Rome, and Dr. V. Nikodem, N.I.H., Bethesda (21)]. The normalization of transfections was carried out with 3 μg of pRSV-CAT (chloramphenicol acethyltransferase). After removal of the precipitate 2nM T3 was applied for 36 hours. CAT and luciferase activities were assayed as described previously (7).

FRTL5 cells were grown as described in Ikuyama et al. (5) Before transfection, FRTL5 were seeded (1,2 10^6 cells per 10-mm tissue culture dish), grown for two days in a medium including six hormones, 6H (7), including TSH. Three days before transfection the cells were grown in a medium with five hormones, 5H, lacking TSH. One day before transfection the cells were returned to 6H medium. The cells were transfected by the calcium phosphate-mediated protocol as described previously (7), with 9 μg of test construct and 3 μg pRSV-CAT. Cell extract were prepared 48 hours after transfection. In the case of 5H experiments the FRTL5 were cultured as described and three days before transfection shifted to 5H medium, lacking TSH, and kept in this medium throughout the experiment.

DNAse 1 protection assay

The TR-α1/Retinoic X Receptor (TR/RXR) complex was a generous gift of Dr. Henk Stunnenberg, EMBL, Heidelberg. It was overexpressed and purified from HeLa cells using the vaccinia virus system (22). Binding reactions are assembled on ice in buffer D (20 mM Hepes pH 7.9, 17% glycerol 5 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EDTA, 0,1% Triton, 2 mM DTT) with 50 ng poly (dI.dC) and the TR/RXR complex.The reaction was started by adding the labeled probe. After 20' on ice the reaction was digested with DNAse 1 according to Lichtsteiner et al. (23). The

digested products were phenol extracted, ethanol precipitated and separated on an 6% sequencing gel. Maxam and Gilbert A+G sequencing reaction of the DNA probe were used to locate the footprinted region.

Promoter mutagenesis

The pGE-T200M2 (pM2) was constructed by using the method of Ho et al. (24) using the pGE-T200 as template and the oligos: 5'-CTAAGCTTTCCAAGGGACCTC CAGTGCTA-3' (A), 5'-GCCGGAGCTCCTAGGCAAGCGGAGCACTT-3' (B) to amplify the template and oligos: 5'-GTCACAGCCCAGGTGAGCCCTCCTCCTTCC-3' and 5'-AGGAGGGCTCACCTGGGCTGTACCTCATC-3' to insert the mutation on the template. The PCR product were digested with Hind III and Sac 1 and cloned in the same restriction sites of pGL2-Basic (Promega, Madison,WI).

Materials

The source of the materials were obtained as reported by Civitareale et al. (7).

RESULTS

In order to investigate the possibility that TR modulates the TSHr promoter activity we transfected a construct containing the minimal TSHr promoter, pGE-T200 (7), driving the luciferase gene, into CV 1 cells, Fig. 1. In this cell line, grown in T3 depleted medium (20), the transfected TSHr promoter shows a basal activity that we have considered arbitrarily as unit. In cotransfections of the pGE-T200 and a vector expressing TRα1, TSHr promoter activity is found to be inhibited only when T3 is added to the medium. This result indicates that the thyroid receptor down-regulates the TSHr promoter in the T3 complexed form. It is worth pointing out that the inhibition we show in this paper is quantitatively similar, about three fold of inhibition, to the down-regulation of the TSHr promoter activity described in FRTL5 cells (reference 4 and this paper, Fig. 3).

Since we found, *in vivo*, an activity of the TR on the TSHr promoter we performed *in vitro* DNA-binding assays to identify the promoter *cis* element. Fig. 2A shows the

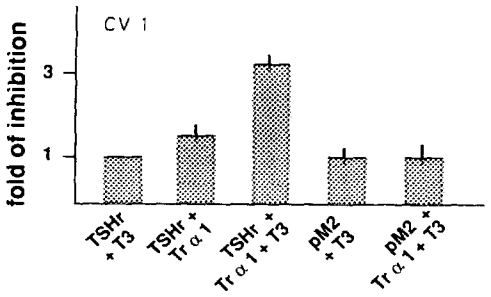

FIG. 1. T3-dependent down regulation of the minimal TSHr promoter in CV 1 cells. The luciferase activity present in extracts of CV 1 cells transfected only with pGE-T200 was arbitrarily set at unity. Cells were cotransfected with an expression plasmid encoding the TRα1 in absence or presence of T3, as indicated. Activities are presented as the mean ± SE for at least three separate experiments; each experiment was done in triplicate.

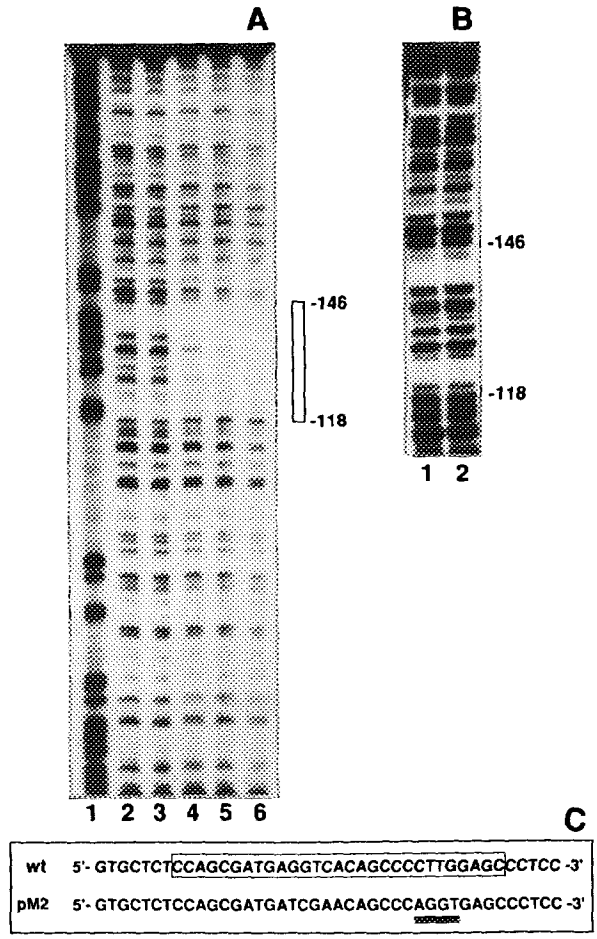

FIG.2. DNAse 1 protection of the recombinant TR on the TSHr promoter. **A**.As substrate we used a Hind III / Sac 1 DNA fragment, derived from the plasmid pGE-T200. This fragment was labeled with [^{32}Pα]CTP and Klenow enzyme and then digested in absence of proteins (lane 2 and 3, two different DNAse 1 digestions) or in presence of increasing amount of recombinant TR (lanes 4,5 and 6). A G+A sequencing reaction of the same fragment was used as size markers (lane 1). Box indicates the protected region and the numbers refer to the boundaries. **B**. As substrate we used a Hind III / Sac 1 DNA fragment, derived from the plasmid pGE-T200M2. This fragment was labeled as described in panel A. Lane 1. DNAse 1 digestion of the described DNA fragment. Lane 2. DNAse 1 digestion of the probe in presence of TR/RXR complex. In this experiment was used the same amount of TR/RXR complex as in lane 6, panel A. **C**. The sequences of the wild type and pM2 of TSHr promoter are reported. The open box indicates the DNAse 1 protected region, the black box underlines the mutagenized sequence.

DNAse 1 protection assay using the recombinant TRα1/RXR complex and the TSHr promoter. A DNAse 1 protection is clearly detected at -118 to -146 base pairs in the TSHr promoter. Within the sequence of the protected region, shown in Fig. 2C, a

	5H	6H
wt	100	40
pM2	100	100

FIG.3. Effect of TSH on the TSHr promoter activity in transfection experiments in FRTL5 cells.
As described in the text, 5H indicates that the cells were grown in a medium with 5 hormones and 6H indicates that TSH was added to 5H. The activity of the wild type TSHr promoter, in cells grown in 5H, is arbitrarily set to 100 %. Activities are presented as the mean for four separate experiments differing by less than 4 %.

half-site TRE consensus sequence, 5'TGAGGTCA3', is present (18). In order to demonstrate that the TR-α1 binding is important for the inhibition of the TSHr promoter acivity, we have mutagenized the proposed binding site. We have constructed a promoter mutant: pGE-T2OOM2 (pM2). The wild type sequence 5'(-125)-CTTG-(-122)3' was changed in 5'-AGGT-3', Fig. 2C. The pM2 mutant shows a promoter activity comparable to the wild type in CV 1 cells and in FRTL5 cells, Fig. 1 and 3. More interestingly the activity of pM2 mutant is not inhibited by T3/TRα1complex in CV 1 cells, Fig. 1, and is not down-regulated by TSH in FRTL5 cells, Fig.3. This loss of down-regulation of the pM2 mutant correlates very well with the loss of TR/RXR complex binding in footprint experiments, Fig.2B, suggesting that the binding of this complex modulates the inhibition observed in the transfection experiments.

DISCUSSION

In the present study we provide evidence that the T3/TR complex is able to down regulate TSHr promoter activity and we show that the TR binds, *in vitro*, to the TSHr promoter. The down-regulation is achieved by TRα1 and only in a hormone bound form. Hence we propose that the TRE half-site, 5'-TGAGGTCA-3', in the TSHr minimal promoter is a nTRE. Since the DNAse 1 protected region, that we show in Fig.2A, extends in both directions from the nTRE half-site, it is likely that this novel nTRE requires further flanking sequences. Thus, in our inhibition mutant, pM2, the mutation is located downstream of the TRE half-site and, as we have shown, it affects both the TR-α1 binding and the promoter activity inhibition. Therefore we suppose that the TRα1 could be complexed with TRAPs (11) also on this promoter. Experiments are in progress to address this point.

Consistent with our finding, it has been proposed that the characteristic of the nTRE is to be a non-palindromic TRE half-site (25). Carr and Wong (25) have used the nTRE of the TSH-β subunit promoter and this nTRE half-site ligated in front of the TK minimal promoter mediates TR down-regulation of this promoter activity in GH3 and in COS7 cells.

Interestingly, the TR binding site in the TSHr promoter overlaps with a CRE-like site already described as a positive and constitutive *cis* element (6). Therefore we could envisage a mutually exclusive binding between a CREB-like positive factor and TRα1. A similar case has been shown on the TSH-β promoter where AP-1 antagonizes the down regulation mediated by TR (26).

In this paper we provide the first molecular evidence for the old hypothesis that thyroid hormone directly inhibits thyroid function (14) and a short loop regulatory mechanism was already proposed (27). Moreover it has also been described, in human thyroid slices, that the T4/T3 had an inhibitory activity on the adenyl cyclase-cAMP system stimulated by TSH (15). More recently, Akiguchi et al. (17) studied the thyroid hormone receptor in FRTL5 cells and found that, in this cell line, there are both, α1 and β1, TR forms but while the β1 form is TSH independent, the α1 form is induced by TSH. This finding supports our hypothesis that TSHr down-regulation, induced by TSH (4), is mediated by the TRα1. The binding of TSH to the FRTL5 plasma membrane induces the TR α1 synthesis thus the thyroid hormone receptor α1, complexed with T3, inhibits the TSHr promoter activity.

The regulatory short loop that we propose in this paper adds to the negative control that T3 plays on TSH gene expression in the pituitary gland and on the TSH-releasing hormone in the hypothalamus to regulate the serum T3 concentration.

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REFERENCES

1. Dumont JE, Lamy F, Roger P, Maenhaut C (1992) Physiol Rev 72, 667-698
2. Vassart G, Dumont JE (1992) Endocr Rev 13, 596-611
3. Nagayama Y, Rapoport B (1992) Mol Endocrinol 6, 145-156
4. Saji M, Akamizu T, Sanchez M, Obici S, Avvedimento E, Gottesman ME, Kohn, LD (1992) Endocrinology 130, 520-533
5. Ikuyama S, Niller HH, Shimura H, Akamizu T, Kohn LD (1992) Mol Endocrinol 6, 793-804

6. Ikuyama S, Shimura H, Hoeffler JP, Kohn LD (1992) Mol Endocrinol 6,1701-1715
7. Civitareale D, Castelli MP, Falasca P, Saiardi A (1993) Mol Endocrinol 7, 1589-1595
8. Oppenheimer JH, Schwartz HL, Strait KA (1994) Eur J Endocrinol 130, 15-24
9. Desvergne B (1994) Mol Cell Endocrinol 100, 125-131
10. Yen PM, Chin WW (1994) Trends Endocrinol Metab 5, 65-72
11. de Groot LJ (1993) Endocrinology 13, 963-964
12. Wood WM, Kao MY, Gordon DF, Ridgway EC (1989) J Biol Chem 264, 14840-14847
13. Carr FE, Burnside J, Chin WW (1989) Mol Endocrinol 3, 709-716
14. Cortell R, Rawson RW (1944) Endocrinology 35, 488-498
15. Takasu N, Sato S, Tsukui T, Yamada T, Furihata R, Makiuchi M (1974) J Clin Endocrinol Metab 39, 772-780
16. Toyoda N, Nishikawa M, Mori Y, Gondou A, Ogawa Y, Yonemoto T, Yoshimura M, Masaki H, Inada M (1992) Endocrinology 131, 389-394
17. Akiguchi I, Strauss K, Borges M, Silva JE, Moses AC (1992) Endocrinology 131, 1279-1287
18. Schrader M, Becker-André M, Carlberg C (1994) J Biol Chem 269, 6444-6449
19. Pfahl M, Tzukerman M, Zhang X-K, Lehmann JM, Hermann T, Wills KN, Graupner G (1990) Methods Enzymol 189, 256-270
20. Samuels HH, Stanley F, Casanova J (1979) Endocrinology 105, 80-85
21. Farsetti A, Mitsuhashi T, Desvergne B, Robbins J, Nikodem V (1991) J Biol Chem 266, 23226-23232
22. Vivanco Ruiz MdM, Bugge TH, Hirschmann P, Stunnenberg H (1991) EMBO J 10, 3829-3838
23. Lichtsteiner S, Wuarin J, Schibler U (1987). Cell 51, 963-973
24. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Gene 77, 51-59
25. Carr FE, Wong NCW (1994) J Biol Chem 269, 4175-4179
26. Wondisford FE, Steinfelder HJ, Nations M, Rodovick S (1993) J Biol Chem 268, 2749-2754
27. Yu S, Friedman Y, Richman R, Burke G (1976) J Clin Invest 54, 745-755