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STUDIES ON THE FUNCTIONAL ACTIVITY OF THE PROMOTER FOR THE HUMAN THYROID PEROXIDASE GENE

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SUMMARY We isolated the initial 1.3 kb of the 5'-flanking region of the human thyroid peroxidase (hTPO) gene, and sub-cloned this fragment into the luciferase reporter gene expression plasmid pA3-LUC. This plasmid contruct (p1.3HTPO-LUC) was stably transfected into FRTL5 rat thyroid cells and NIH-3T3 fibroblasts. Promoter activity was detected in 3 of 8 FRTL5 stable cell lines obtained. TSH, dBcAMP and phorbol ester did not alter TPO promoter activity. TPO promoter activity was also expressed in 4 of 5 NIH-3T3 stably-transfected cell lines, and this activity was also not altered by dBcAMP and phorbol ester. These data support the emerging concept that the TPO gene is not transcriptionally regulated by TSH and cAMP.

Thyrotropin (TSH) stimulation of thyroid cells increases the activity of TPO, the primary enzyme involved in thyroid hormone synthesis (1,2). This action of TSH is associated with an increase in the steady state levels of TPO mRNA in all species tested (3-6), a process mimicked by cAMP (3-5). There is debate as to whether TSH produces this effect by a transcriptional or non-transcriptional mechanism. Nuclear run-on transcriptional studies show no transcriptional activation of the TPO gene by TSH in FRTL5 rat thyroid cells (4,7), but transcriptional activation by TSH is seen in dog thyroid cell primary cultures (8). In contrast to these transcriptional studies, there is no report on the functional activity of the promoter in the TPO gene. In the present study we report the presence of constitutive promoter activity in the initial 1.3 kb of the hTPO 5'-flanking region. The TPO promoter functional activity in this region is not altered by TSH or cAMP. These data support the evidence that TSH

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Abbreviations: TSH: thyrotropin; hTPO: human thyroid peroxidase; PCR: polymerase chain reaction; dBcAMP: dibutyryl cyclic AMP.

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regulates TPO gene expression, at least in FRTL5 cells, in a non-transcriptional manner.

METHODS AND MATERIALS

Construction of p1.3HTPO-LUC: A human genomic DNA library (9) was screened (10) with a 62 bp Dde I fragment from the 5'-end of human TPO(11). Confirmation of the hTPO 5'-flanking region in clone GC1 was obtained by nucleotide sequencing (12). After subcloning into pUC18, a 1.3 kb Sac I (blunted)-Hind III fragment was ligated into the the Sma I-Hind III sites of pA3-LUC (13) (kindly provided by Dr. William M. Wood, Denver, CO). Nucleotide sequencing confirmed the correct orientation and splicing of the fragment, with the TPO 5'-flanking region extending downstream to position +15 in the untranslated first exon.
Cell transfections: FRTL5 rat thyroid cells (14) and NIH-3T3 fibroblasts were cultured as previously described (15). Cells were co-transfected (16) with 20 ug of p1.3HTPO-LUC, and 2 ug pSV2-neo DNA (17). G418 (400 μg/ml; GIBCO Laboratory; Grand Island; NY) resistant clones were selected with cloning cylinders and expanded. We studied multiple, individually-selected, stable cell lines rather than pooled clones, because the very long time (months) required to obtain stable transfectants (4 d doubling time for FRTL5 cells) made repeated transfections impractical. NIH-3T3 cells were further subcloned by limiting dilution. As negative controls, cells were also transfected with pA3-LUC. For positive controls, cells were transfected with Rous sarcoma virus (RSV) promoter-driven luciferase constructs (pRSV180-LUC and pRSV400-LUC; also from Dr. William M. Wood). Confirmation of the hTPO transgenome was obtained by PCR (18), using genomic DNA (10) and two oligonucleotide primers; 5'-TCCAGGAACCAGGGCGTATCTCTT extending upstream from position 165 in the luciferase gene (13), and TGCATGCTGGTGAACACACA extending downstream from position -520 in the human TPO 5'-flanking region (see Fig. 1). PCR conditions (30 cycles) were: 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. Luciferase activity was measured using a luminometer (19).

RESULTS

The nucleotide sequence of the human TPO 5'-flanking region (up to 560 bp upstream of the transcription initiation site) was determined (Fig. 1). After the completion of this work, Kimura et al. reported nucleotide sequence data on this region (20) that are identical to our findings. There are no regions, within the TPO 5'-flanking region that was sequenced, that are homologous to known response elements for trans-acting factors (21).

FRTL5 cells are a very difficult line to study because of their low transfection efficiency (15), but they are the only continual thyroid cell line available for tissue-specific analysis of TPO promoter functional activity. We elected to stably-transfect FRTL5 cells with p1.3HTPO-LUC after transient transfection studies revealed TPO promoter activity to be too weak to provide conclusive information (data not shown).

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                                                          -500
tctttgatgggtgcatgatgaggattgaggggagaatgcatgctggtgaacacacactgaactgtgctca

ttttcacctcaacaaattggaaaaaagtgtactttattcacctcttagtgataagaaaactaggggctt

                  -400
ggggacaggaagcaaaaggctcagagttgtagagcgagtcatcggtgggcctgggagctgcacccaaccc

                                                      -300
aatcctcggaagattaacagccccttttcacagggtatttaactgtgaaagtaagagggaggaaaatgc

aagtgtcacaagtctggatttagttggtttattctttctccctgtataatttttcccctcttcttcctaa

        -200
ctcattcggccagaggctggactgcatgtggaccccgatgacatggcactttgtttctgaccagtcagga

                                          -100
cacacaagaggcccggcgcaaacacaacaaagcccgcagacattctgtccccacgaagaacggacgccac

tcgacttcctagcatcttgacgggctatccaagcgcagagtcagtttataaggtgggtaaccaagtccct

+1
gGAAGGCAATTGAGGCGCCCATTTCAGAAGAGTTACAGCCGTGAAAATTACTCAGCAGTGCAGTTGGCTG

                                  100
AGAAGAGGAAAAAAGgtcaggttgtaaagctt
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Figure 1: Nucleotide sequence of part of the 5'-flanking region of the human TPO gene. Bases are numbered from the first nucleotide of the transcription initiation site (+1). The 84 bp first (untranslated) intron is displayed in capital letters. The TATA box motif is underlined.

We studied a total of 53 stably-transfected cell lines (26 FRTL5 and 27 NIH-3T3) (Fig. 2). Three of 8 of p1.3HTPO-LUC FRTL5 clones expressed luciferase activity, and two of these were exceptionally strong (>5000-fold above basal levels). A number of NIH-3T3 cells stably-transfected with p1.3HTPO-LUC also expressed TPO promoter activity (Fig. 2). The presence of TPO 5'-flanking region-luciferase transgenomic chimera was confirmed in these cell lines by PCR (data not shown). RSV promoter-driven luciferase activity was also detected in both cell types, but was relatively stronger in the NIH-3T3 cells (Fig. 2).

Stably-transfected FRTL5 and NIH-3T3 cells lines expressing TPO promoter activity were then studied with respect to promoter responsiveness to TSH, cAMP and phorbol ester (TPA) stimulation. After a 6 d period of quiescence in TSH-free medium, TSH stimulation had no activity on the constitutive TPO promoter activity in two lines of FRTL5 cells stably-transfected with p1.3HTPO-LUC (Fig. 3). A similar lack of effect was observed with dibutyryl cAMP and the phorbol

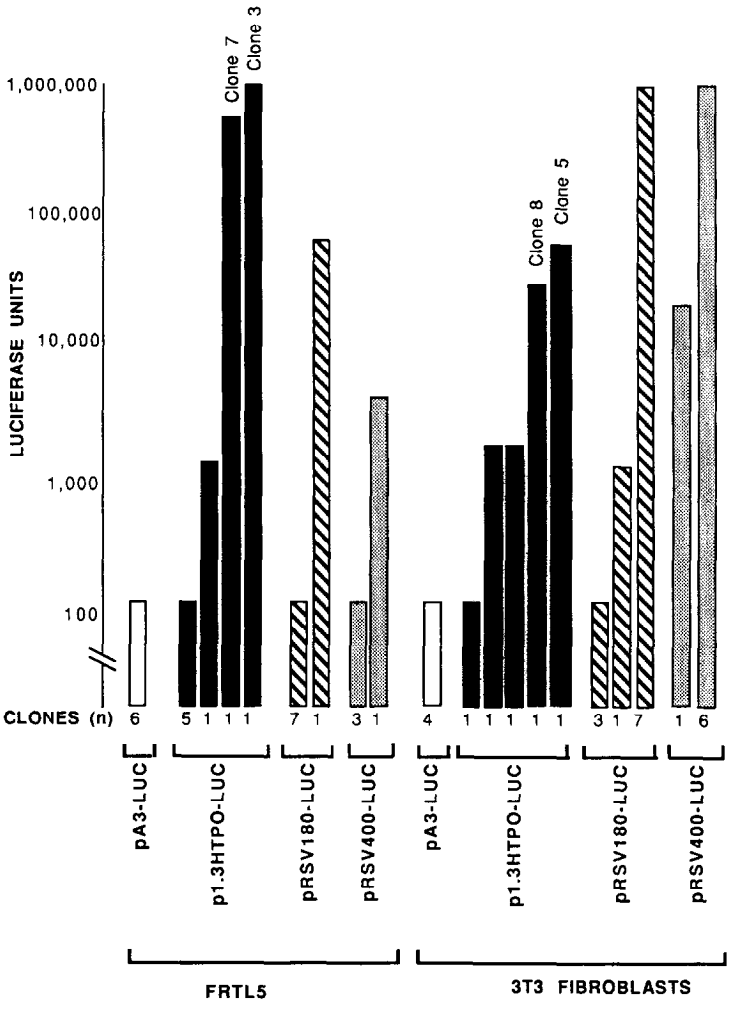

Figure 2: Analysis of FRTL5 rat thyroid and NIH-3T3 mouse fibroblast cell lines stably-transfected with the indicated plasmids (Methods). Clones were screened for luciferase activity (counts read on a luminometer) when they reached confluence in 100 mm diameter dishes. Note the logarithmic scale used because of the very wide range of activities among the different clones. The number of clones studied is indicated below each bar.

ester, 12-O-tetradecanoyl phorbol 13-acetate (TPA) (Fig. 3). Because we found that the TPO promoter could be expressed in NIH-3T3 cells, and might therefore be influenced by post-receptor signalling mechanisms, we also examined the effect of dBcAMP and TPA on TPO promoter activity in these cells. As in FRTL5 cells, the TPO promoter was unresponsive to these agents (Fig. 4).

DISCUSSION

There are no previous reports on the functional activity of the promoter for the TPO gene. The present study demonstrates TPO promoter activity within

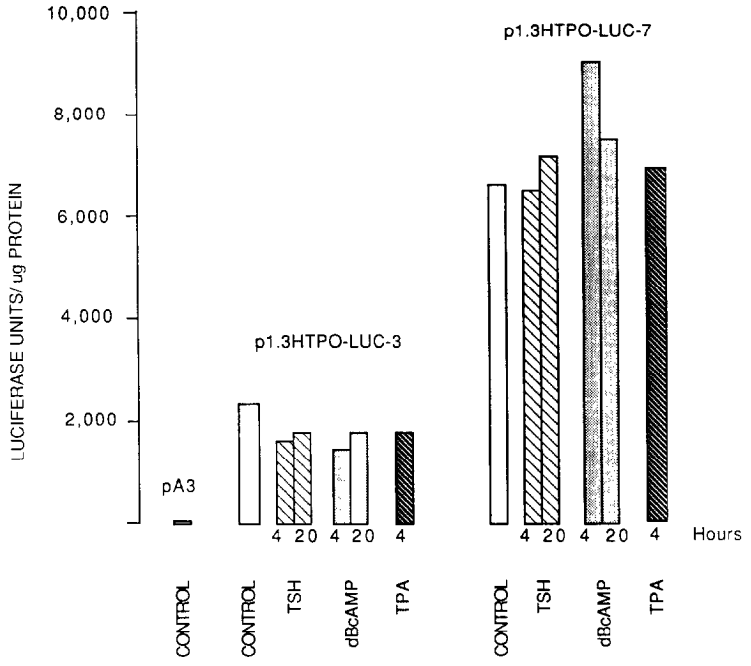

Figure 3: Effect of TSH, dBcAMP and TPA on TPO promoter activity in stably-transfected FRTL5 cells. Clones 3 and 7, stably transfected with p1.3HTPO-LUC, were cultured in TSH-free medium for 6 days, and were then stimulated with TSH (5 mU/ml), dBcAMP (1 mM) and TPA (50 ng/ml) for the indicated times. Similar results were obtained in a separate experiment.

the initial 1.3 kb of the 5'-flanking region of this gene, however this region does not appear to have a TSH, cAMP or phorbol ester response element. Unlike with the positive demonstration of a hormone response element in a gene, it is more difficult to conclude (see below) that such an element is absent. Nevertheless, the apparent lack of hormone responsiveness within the initial 1.3 kb of the TPO 5'-flanking region is consistent with lack of response to TSH stimulation in run-on transcription assays using nuclei prepared from FRTL5 cells (4,7). The evidence so far available for the FRTL5 cell line, unlike dog thyroid cell primary cultures (8), therefore favors the non-transcriptional regulation by TSH of TPO mRNA levels.[2]

[2]Since submission of our manuscript, Abramowicz, Vassart and Cristophe have reported that the human thyroid peroxidase promoter is activated by TSH and cAMP (Biochem. Biophys. Res. Comm. 166:1257-1264). While these data may appear to be contradictory with those of the present study, the hTPO promoter data in each study are consistent with the nuclear run-on transcription data previously reported (4,7,8). It is therefore possible that different transcriptional responses to TSH may exist in different thyroid cell models.

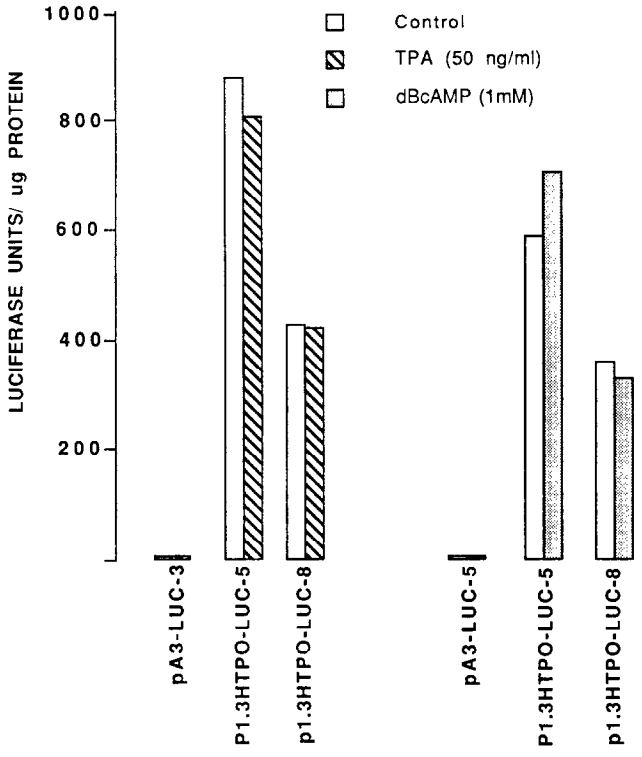

Figure 4: Effect of dBcAMP and TPA on TPO promoter activity in stably-transfected NIH-3T3 cells. Clones 5 and 8, stably transfected with p1.3HTPO-LUC, were incubated for 20 h with TPA (50 ng/ml) or dBcAMP (1 mM) added to the culture medium.

Other explanations clearly may account for the apparent lack of TPO promoter response to TSH or cAMP stimulation. Although most of the hormone response elements so far identified would be included within the initial 1.3 kb of the 5'-flanking region, such elements may be present much further upstream, or even within exons, introns, or in the 3'-flanking region. Whether or not a hormone response element is present beyond the confines of this 1.3 kb region will require additional study. Another possible explanation for the lack of apparent TSH responsiveness is that TSH may induce the transcription of a luciferase mRNA with an altered cap site. It is also possible that the site of insertion of the TPO 5'-flanking region transgenome may contribute to the lack of TSH responsiveness. However, this possibility is less likely in view of the lack of effect observed in multiple cell lines.

It is of interest that the promoter for TPO, a gene expressed only in thyroid cells in vivo, is also functional in cultured fibroblasts, although

perhaps to a lesser degree. This finding suggests that a tissue-specific regulatory element may lie outside the 1.3 kb of the 5'-flanking region studied. Alternatively, NIH-3T3 cells, an immortalized line, may lack a transcriptional repressor. Although the same criticism may be applied to FRTL5 cells, these cells are sufficiently differentiated so that they retain the unknown factors presumably needed for the TPO mRNA response to TSH stimulation (4).

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