Discrete Positive and Negative Thyroid Hormone-responsive Transcription Regulatory Elements of the Rat Growth Hormone Gene*

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We have recently shown that a thyroid hormone-responsive transcription stimulatory element exists in the 5'-flanking DNA near the rat growth hormone (rGH) gene (Crew, M. D., and Spindler, S. R. (1986) J. Biol. Chem. 261, 5018-5022). Progressive deletion-transfection analysis of the 5' end of the gene has led to the identification of two genetic elements responsive to thyroid hormone. The first of these is a thyroid hormone-responsive transcription stimulatory element, or TSE. The TSE induced a thyroid hormone-dependent induction-attenuation transcription cycle similar to that of the natural rGH gene. Deletion of sequences between positions -254 and -241 in the rGH 5'-flanking DNA eliminated TSE activity. The second regulatory element is a thyroid hormone-responsive transcription inhibitory element (TIE). When this element was active, thyroid hormone strongly but transiently inhibited rGH promoter utilization. Deletion of sequences between nucleotides -46 and -21 abolished the effects of the TIE.

To determine whether the TSE and TIE are enhancer-like, we ligated various regions of rat growth hormone 5'-flanking DNA containing these elements to a chimeric test gene containing the Herpes simplex virus thymidine kinase promoter. Thyroid hormone activated heterologous promoter utilization when a rat growth hormone 5'-flanking DNA fragment containing the TSE (-520 to -115) was linked in cis, regardless of the distance or orientation of the TSE with respect to the promoter. These data suggest that the TSE is a thyroid hormone-dependent enhancer. In contrast, when the TIE was placed immediately 5' to the thymidine kinase promoter, transcription was not effected by 3,5,3'-l-triiodothyronine, suggesting that the TIE is not enhancer-like.

The rat growth hormone (rGH) gene provides an excellent model for studies of eucaryotic gene regulation. Its transcription is cell-type-dependent (1, 2) and regulated by thyroid and glucocorticoid hormones in vivo (3) and in cultured rat pituitary tumor cells (GC cells, Ref. 4). Thyroid hormone (3,5,3'-L-triiodothyronine, T₃) stimulates transcription of the gene in a receptor-dependent manner, suggesting that the occupied receptor interacts directly with the gene to stimulate initiation (5). Consistent with this hypothesis, T₃ induces promoter-proximal local alterations in chromatin structure which are temporally coincident with the T₃-induced changes in transcription (6).

To elucidate the mechanism by which T₃ regulates rGH transcription, we analyzed the effects of T₃ on promoter utilization in GC cells stably transfected with chimeric genes composed of various regions of rGH 5'-flanking DNA linked to the dominant selectable marker gene, neo (2). The use of stable transfections allows kinetic studies of the expression and regulation of these genes without the uncertainties associated with exogenous DNA uptake and loss inherent in transient transfection studies. Such considerations are especially important in the analysis of a cyclic regulatory phenomenon such as T₃ action (2, 3, 5). Furthermore, T₃ has a profound effect on the general rate of metabolism and growth of GC cells (7). Thus, the hormone may affect the uptake and stability of transiently transfected DNA. In contrast, the principal problem associated with stable transfections, "position effects," can be negated by analysis of pools of transfectants obtained with each construct (2).

Accurate, T₃-regulated transcriptional initiation has been obtained on the rGH promoter using approximately 1760 base pairs (bp) of rGH 5'-flanking DNA (2, 8). We also have shown that chimeric genes which share 235 base pairs of rGH 5' DNA are capable of directing accurate, T₃-regulated transcriptional initiation identical to that of the endogenous gene (2). In the course of investigating the transcriptionally active sequences flanking the gene, we have discovered two discrete T₃-responsive regions. One of these regions stimulates transcription from cis-linked promoters in an enhancer-like manner. The other element inhibits transcription initiated from the rGH TATAA sequence, but does not inhibit initiation when linked 5' to the thymidine kinase promoter.

MATERIALS AND METHODS

Construction of Chimeric Genes—Progressive 5' deletion of rGH flanking DNA was accomplished by linearization of prGH3570neo (Fig. 1A; Ref. 2) with HindIII digestion for various lengths of time with exonuclease Bal31, ligation of synthetic EcoRI linkers, EcoRI digestion, and circularization of the plasmid with T4 DNA ligase (9). ptkneo was constructed by ligating the 180-bp SalI to XhoI fragment of ptkCAT (constructed by R. J. Miksic, Institute of Cell and Tumor Biology, German Cancer Research Center, Heidelberg) to SalI-cleaved pUCneo (2). Genes GH-520/-115tkn and GH-115/-520tkn were constructed by cleavage of prGH530neo (2) with XhoI, limited digestion with exonuclease Bal31, ligation with HindIII linkers, HindIII digestion, and ligation to HindIII-cleaved, dephosphorylated ptkneo. GH-520/-115tkn and GH-115/-520tkn were constructed by cleavage of prGH530neo (2) with XhoI, limited digestion with exonuclease Bal31, ligation with HindIII linkers, HindIII digestion, and ligation to HindIII/NcoI fragment of ptkneo. The end points of all deletions were determined by DNA sequencing (10, 11).

Cell Culture and Transfection—GC cells were cultured and transfected in DME-H21 medium containing 10% fetal calf serum as previously described (2, 5). This medium contains approximately 0.2 nm T₃. The T₃ receptor is approximately 50% occupied at this hormone concentration. Transfections of cell monolayers were performed using the CaPO₄ DNA coprecipitation method followed by

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The abbreviations used are: rGH, rat growth hormone; T₃, 3,5,3'-L-triiodothyronine; bp, base pair(s); TSE, T₃ stimulatory element; TIE, T₃ inhibitory element; rGH, human growth hormone.
**Table I**

**T_{3} response of chimeric genes**

Autodisplaced Northern and dot blots of RNA obtained from two inductions performed with colonies from each of two separate transfections of each construct were scanned, digitized, and normalized to the level of actin-tubulin RNA in each sample. Shown is the average fold T_{3} induction or inhibition of neo RNA.

<table>
<thead>
<tr>
<th>Gene construct</th>
<th>Induction(^{a})</th>
<th>Inhibition(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>prGH1760neo</td>
<td>2.7 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>prGH308neo</td>
<td>4.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>prGH300neo</td>
<td>2.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>prGH285neo</td>
<td>2.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>prGH254neo</td>
<td>2.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>prGH241neo</td>
<td>1.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>prGH162neo</td>
<td>2.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>prGH145neo</td>
<td>4.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>prGH134neo</td>
<td>2.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>prGH61neo</td>
<td>4.8 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>prGH46neo</td>
<td>7.9 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>prGH21neo</td>
<td>1.1 ± 0.2</td>
<td></td>
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\(^{a}\)The normalized intensity of the neo signal obtained using RNA from T_{3}-treated cells/the normalized intensity of the neo signal obtained using RNA from uninduced cells.

\(^{b}\)The normalized intensity of the neo signal obtained using RNA from uninduced cells/the normalized intensity of the neo signal obtained for T_{3}-treated cells.

**Results**

Chimeric genes consisting of various lengths of rGH 5'-flanking DNA linked to the bacterial antibiotic resistance gene neo were constructed (Fig. 1A) and stably transfected into GC cells. The neo transcripts in cell cultures derived from pooled colonies of homologous transfecteds were analyzed by Northern blotting (Fig. 1B). Table I summarizes the results obtained from two separate transfections of each construct.

Deletion of 5' sequences upstream from position -254 had little effect on the T_{3} inducibility of the rGH promoter (Fig.

**Fig. 1.** The effect of T_{3} on the expression of constructs with rGH 5' deletions. A, structure of deleted constructs. The constructs shown are named according to the length of rGH 5'-flanking DNA they contain. For example, prGH285neo is composed of 285 nucleotides of rGH 5'-flanking DNA, and the neo gene-coding sequence and SV40 virus RNA processing signals of pSV2neo (12). rGH DNA is indicated by the open regions, neo sequences are stippled, and plasmid and polylinker sequences are thin lines. B, relative promoter activity of the deleted constructs. The rGH 5' end point of each deleted selection of stable transfectants in 400 µg/ml antibiotic G-418 for at least 15 days (2).

**RNA Isolation and Analysis.—** Ten to 100 G-418-resistant colonies obtained with each construct were pooled and grown to confluence in media lacking G-418. Cells were subcultured, deinduced for 3-5 days in media depleted of thyroid and steroid hormones (hypomedium; Refs. 2, 5), and treated with 10 nM T_{3} for the indicated times. RNA was isolated in the presence of 10 mM vanadyl-ribonucleoside complex as recommended by the supplier (Bethesda Research Laboratories). Total cytoplasmic RNA was analyzed by Northern blotting (9) and probed sequentially with the coding regions of the neo (12), rGH (13), and actin-tubulin genes (14). Samples were also analyzed by dot blotting. Autoradiograms were scanned and the results digitized using an LKB ultra laser scanner. Neo and rGH RNA levels were normalized to those of actin-tubulin, which varied by <30% between samples. There was no significant difference in the level of actin-tubulin RNA present in RNA preparations from T_{3}-treated or -untreated cells during 48 h of hormone treatment.

**Deletion of 5' sequences upstream from position -254 had little effect on the T_{3} inducibility of the rGH promoter (Fig.
**T3-responsive Transcription Regulatory Elements**

Fig. 2. Kinetics of the T3 response in transfected cells. A, Northern blot. RNA was purified from prGH285neo-, prGH241neo-, and prGH134neo-transfected cells which had been deinduced for 3 days in hormone-depleted medium and induced with T3 for the indicated times. The blot was serially probed with neo and rGH and kal-tubulin cDNA. These RNA are indicated by the labeled arrows. Lanes 1–5 represent the results obtained with prGH285neo-transfected cells; lanes 6–10 represent prGH241neo-transfected cells; and lanes 11–15 represent prGH134neo-transfected cells. Cells were induced with T3 for 0 (lanes 1, 6, 11), 4 (lanes 2, 7, 12), 8 (lanes 3, 8, 13), 12 (lanes 4, 9, 14), and 24 h (lanes 5, 10, 15). B, Kinetics of neo RNA induction by T3. The Northern blot results shown in A were quantitated and normalized to the level of kal-tubulin RNA observed, cannot explain either the induction or repression of neo RNA found with these constructs. The expression and T3 regulation of the natural rGH gene was normal in all the cell lines obtained (Fig. 1C and data not shown), indicating that the results are not due to abnormal T3 responsiveness in some cell lines. These data confirm that both T3-stimulated and T3-inhibited transcription of rGH 5′-flanking sequences present in the constructs.

Studies performed to characterize the kinetics of the TSE and TIE response are shown in Fig. 2. Cell lines transfected with prGH285Neo, prGH241Neo, or prGH134Neo were hormonally deinduced and subsequently induced for various lengths of time with T3. As shown in Fig. 2, A (lanes 1–5) and B, prGH285Neo transcription responded to hormone addition with an induction-attenuation transcription cycle characteristic of the intact promoter (2, 4). In contrast, a slight inhibition of neo expression was found after T3 addition to prGH241Neo-transfected cells (Fig. 2, A, lanes 6–10 and B).

In prGH134Neo-transfected cells, neo RNA levels were higher in the absence of T3 (Fig. 3A, lane 11) and were of purified RNA were probed for neo, rGH, and kal-tubulin sequences. Only the results obtained with the neo probe are shown. The kal-tubulin RNA varied by less than 20% between these samples, and the rGH RNA was induced normally.
A ptkneo

HindIII SalI (Xho/SalI)

HindIII PstI

HindIII

GH -520/-115tk

GH -115/-520tk

GH-134/+11tkn

B

ptkneo

GH-520/-115tk

GH-115/-520tk

GH-134/+11tkn

neo

Fig. 4. T3 regulation of chimeric genes containing the thymidine kinase promoter. A, structure of rGH-thymidine kinase-neo chimeric genes. rGH DNA is hatched, thymidine kinase sequences are black, neo-coding sequences are stippled, and plasmid and polylinker sequences are thin lines. The direction of the rGH 5'-flanking DNA relative to the transcription start site in the natural gene is indicated by the arrowhead. The numbers below the rGH regions refer to the positions of these sequences relative to the transcription start site of the natural gene. Relevant restriction nuclease cleavage sites are shown. B, effects of T3 on neo RNA levels in transfected cells. Northern blots were probed sequentially with neo, rGH, and k01-tubulin cDNA. Shown are the levels of neo RNA before (lanes 1, 3, 5, and 7) and 8 h (lanes 2, 4, and 6) or 6 and 12 h (lanes 8 and 9, respectively) after addition of T3 to hormonally deinduced cells. k01-Tubulin RNA varied in each induction by 30% or less. The endogenous rGH gene induced normally in each case.

Plasmid GH-520/-115tkn contains rGH 5'-flanking DNA sequences from -520 to -115 linked to the thymidine kinase promoter and neo gene-coding sequences (Fig. 4A). The thymidine kinase promoter region used lacks an enhancer (18, 19).

To test for enhancer-like characteristics, chimeric genes were constructed containing various rGH gene 5'-flanking sequences linked to the viral thymidine kinase promoter and neo gene-coding sequences (Fig. 4A). The thymidine kinase promoter region used lacks an enhancer (18, 19).

If the action of the TSE involves enhancer-like mechanisms, it should activate transcription from a heterologous promoter independent of position or orientation (16, 17). Likewise, if the TIE behaves as an enhancer, it should inhibit initiation from a heterologous promoter in a similar fashion. To test for enhancer-like characteristics, chimeric genes were constructed containing various rGH gene 5'-flanking sequences linked to the viral thymidine kinase promoter and neo gene-coding sequences (Fig. 4A). The thymidine kinase promoter region used lacks an enhancer (18, 19).

Plasmid GH-520/-115tkn contains rGH 5'-flanking DNA sequences from -520 to -115 linked to the thymidine kinase promoter in the same orientation as in the natural rGH gene. Plasmid GH-115/-520tkn contains the same rGH region linked in the opposite orientation and separated by 822 bp of vector DNA from the thymidine kinase promoter (Fig. 4A). Thus, the TSE in these constructs is placed at different distances in either orientation relative to the heterologous thymidine kinase promoter. These genes lack the rGH TA-TAA, GAAT, and TIE sequences.

Fig. 4B shows the effects of T3 on the level of neo transcripts after stable integration of these genes into the GC cell genome. T3 induced a 4-6-fold increase in both GH-520/-115tkn and GH-115/-520tkn promoter utilization after 8 h (Fig. 4B,
compare lanes 3 and 4, and lanes 5 and 6). In contrast, the hormone produced no change in the expression of transfected ptkneo (Fig. 4B, lanes 1 and 2). Thus, the rGH TSE functions in both orientations and at variable distances when cis-linked to a heterologous promoter. These results suggest that the TSE is a T3-dependent enhancer.

Plasmid prGH-134/+11tkn contains the rGH sequences which mediate TIE activity linked to the 5' end of the thymidine kinase promoter in the same orientation with regard to the promoter as in the natural gene. As shown in Fig. 4B, neo mRNA levels in prGH-134/+11tkn-transfected cells are not T3-responsive (compare lane 7 with lanes 8 and 9). We do not know yet whether elements of the thymidine kinase promoter suppress or override the TIE, or whether the thymidine kinase promoter is not affected by the TIE and is cis-dominant over the rGH promoter. What is clear from these data is that the TIE does not behave as predicted for a T3-inhibited, enhancer-like element.

**Discussion**

The data presented in this report demonstrate the existence of two discrete T3-responsive elements in rGH 5'-flanking DNA. One of these elements, termed a TSE, mediates T3-dependent stimulation of transcription in an enhancer-like manner. Sequences essential to full TSE activity lie between -254 and -241. The second element, termed a TIE, mediates T3-dependent inhibition of transcription. TIE activity requires rGH flanking DNA between -46 and -21. The TIE is not enhancer-like in its properties. The response of these elements to T3, like the response of the natural rGH gene, is transient in nature. Deletion of sequences between -241 and -162 appears to activate the TIE. Thus it is possible that genetic elements regulating TIE activity exist in this region of flanking DNA. Alternatively, sufficient TSE activity may remain after deletion to -241 to mask transcriptional inhibition by the TIE.

In our previous work, construct prGH41460neo, containing rGH 5'-flanking sequences from +11 to -235 linked to a 57-base pair fragment of distant 5'-flanking DNA, responded to T3 with an enfeebled, positive transcription cycle (2). In the studies reported here, essentially all positive T3 responsiveness was lost after deletion of flanking DNA to nucleotide -241. The most likely explanation for the discrepancy between these studies is that the distant 5'-flanking DNA present in prGH41460neo is either itself a weak TSE, or fortuitously reconstructs part of the deleted sequences required for minimal TSE activity. We have sequenced this 57-bp fragment, and a 10-nucleotide sequence present between -241 and -254 is also present within the 57-bp fragment (data not shown).

Examination of chromatin structure surrounding the rGH gene has shown that occupancy of the T3 receptor is accompanied by a transient increase in the DNase I sensitivity of a site centered at position -170, and the appearance of a site centered at position -25 (6). The locations of these sites correlate well with the position of the TSE and TIE, within the resolution of DNase I-hypersensitive site mapping. The transient appearance of the sites correlates well with the transient transcription cycle induced by T3. These data and the dose-response data presented here suggest that occupied T3 receptor interacts with the TIE and TSE.

Larsen et al. (20), using transient transfection assays, recently found that deletion of rGH sequences between -237 and -202 resulted in a 3-fold decrease in T3 inducibility. They did not test constructs deleted between position -237 and the EcoRI site at approximately -1760. Thus, their data place the 5' boundary of the TSE in the same vicinity identified by us previously (2) and here. Larsen et al. (20) did not detect the activity of the TIE when they examined expression of the chloramphenicol acetyltransferase reporter sequence 24 h after T3 addition. However, the TIE response occurs soon after T3 addition, and disappears by 24 h. Thus, Larsen et al. (20) may have temporally overlooked the TIE response.

Larsen et al. (20) argue that analysis of T3 responsiveness using stably transfected genes may bias the results obtained by selecting for integration into "inherently T3-responsive areas of the genome." However, chromatin position effects can explain the results presented here only if different rGH flanking DNA regions mediate sequence-dependent, high-efficiency targeted integration into qualitatively different T3-responsive and -nonresponsive chromatin domains. Such effects appear highly unlikely.

Cattini et al. (21) have recently presented evidence obtained using stable transfections that expression of the human growth hormone (hGH) gene is negatively regulated by T3. Consistent with their findings, the region of the rGH gene containing the TIE is highly homologous to the cognate hGH 5'-flanking region. It is possible that the hGH gene, like the rGH gene, contains a TIE in this region. It is also possible that a TIE exists for the hGH gene but that is in a different position on the hGH gene than on the rGH gene and may not have been present in the constructs tested by Cattini et al. (21). Alternatively, a TIE may have been lost or was not introduced into the vicinity of the GH gene during human evolution.

The physiological role of the rGH TIE is not yet known. In view of the central position that growth hormone plays in regulating the growth, development, and metabolism of vertebrates, it is likely that the TIE serves a physiological function. Since the TIE co-exists with a TSE in the rat genome, it is likely that TIE activity is regulated by additional hormonal factors.

**References**