Characterization of an Unusual Thyroid Response Unit in the Promoter of the Human Placental Lactogen Gene*

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The human placental lactogen B (hCS-B) promoter activity is strongly stimulated by thyroid hormones in the rat pituitary GC cell line. The minimal DNA sequence required for stimulation, as determined by transfection with 5' and 3' deletion mutants, spans 67 base pairs, from coordinate -97 to -31. DNase I footprinting experiments show that this thyroid response unit includes two adjacent binding sites: one for the thyroid receptor (-67/-41), the other for the pituitary-specific factor GHFl (-95/-68). Neither region alone is sufficient to confer thyroid responsiveness. The thyroid receptor binding element (TBE) does not contain any repeats or palindromes but is composed of two different domains, one of which is very similar to the half-palindromic motif described by Glass et al. (Glass, C. K., Holloway, J. M., Devary, O. L., and Rosenfeld, M. G. (1988) Cell 54, 313-323). The other is very rich in purine. The normal human growth hormone (hGH-N) promoter, which is 94% similar to the hCS-B promoter, differs from its hCS-B counterpart precisely in this TBE. This difference may explain the opposite 3',5'-triiodothyronine (T3) regulation of these two genes.

The thyroid hormones exert their effects mainly by regulating transcription of specific genes. They interact specifically with nuclear receptors which then bind to regulatory DNA sequences of the target genes (reviewed in Ref. 2). Recent cloning of the genes encoding thyroid hormone receptors (3, 4) has revealed high homology to steroid, vitamin D, or retinoic acid receptors, thus placing these receptors in the nuclear hormone receptor superfamily (5, 6).

However, as compared with the situation for steroid hormones, much less is known about the thyroid hormone-responsive elements (TRE) that mediate thyroid hormone regulation. The few putative TREs defined to date, by transfection with deletion mutants and/or in vitro binding analysis, often contain quite different sequences and make it difficult to define a general consensus sequence. Recently, Brent et al. (7) have proposed a T3-receptor-binding half-site, AGGTCAAA, at least two copies of which are required for a T3 response. In some reported TREs, however, this consensus is either absent or present only once (8-10). It is necessary to seek other TREs in order to define a general consensus sequence. Cloning of T3 receptor genes and their overexpression in eukaryotic systems (11, 12) will probably greatly facilitate this research. Indeed, availability of nuclear extracts highly enriched in thyroid hormone receptor protein allows footprinting experiments and thus identification of thyroid receptor binding sites. Such studies have been greatly hampered previously by difficulties in receptor purification.

The human growth hormone (hGH) and placental lactogen (hCS) genes are an attractive model for studying thyroid hormone regulation of gene expression. Their 500-bp promoters, although presenting 94% similarity, are regulated in opposite ways by T3 in rat pituitary GC cells: hGH-N promoter activity is inhibited by T3 (13), whereas hCS-A promoter activity is stimulated (14). Although endogenous hCS (A and B) gene expression is restricted to the placenta, numerous studies have shown that the hCS-A and hCS-B promoters are active in GC cells (14-16). This can be explained by the fact that these two promoters bind ubiquitous factors like SP1 but also the pituitary-specific GHFl factor (16, 17).

In this study, we show that, like hCS-A, the highly (99%) similar hCS-B is strongly stimulated by T3 in GC cells. By transfecting with 5' and 3' deletions of hCS-B promoter linked to a reporter gene, we have identified the minimal sequence required for T3 induction. Using the α thyroid receptor overexpressed in the vaccinia virus system (11, 12), we have precisely defined, by DNase I footprinting, the sequences in the hCS-B and hGH-N promoters that interact with the thyroid receptor.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Plasmids rGH50CAT (18), TK105CAT (= pBLCAT2; (19)), and hGH496CAT (20) have been described elsewhere. The RSvamp plasmid contains the Rous sarcoma virus enhancer/promoter inserted upstream from the β-lactamase RTEm (Amp' gene, followed by the SV40 splicing and polyadenylation signals) (21).

The hCS2289CAT plasmid, containing 2289 bp of the human hCS-B gene 5'-flanking region and 6 bp of the transcribed sequence fused to the coding sequence of the bacterial chloramphenicol acetyltransferase (CAT) gene, was constructed as follows. A 2.3-kilobase pair BamHI 5'-flanking fragment of the hCS-B gene (from coordinates -2289 to +6) was cloned into the BamHI site of pA10CAT3. pA10CAT3 is identical to pA10CAT2 (22) except that the HindIII site has been replaced by a BglII site. A clone containing the insert
in the correct orientation was selected by restriction analysis. All the following 5' deletion constructs were made in a modified pBLCAT3(19) plasmid (pBLNHCAT3) where a cryptic NF1 binding site located upstream from the polylinker2 has been deleted. For that purpose, pBLCAT3 was digested by NdeI and HindIII, blunt-ended with the Klencow fragment of DNA polymerase I, and recircularized by T4 DNA ligase. The HindIII site was thus restored. hCS493CAT was constructed by subcloning the EcoRI(-493) (blunt-ended)-HindIII(+6) fragment of the hCS-B gene into the SphI (blunt-ended)/BamHI site of pBLCAT2 (19).

To construct the 5' deletion mutants hCS295CAT, hCS212CAT, hCS171CAT, hCS160CAT, hCS129CAT, and hCS83CAT, plasmid hCS493CAT was digested by HincII, DraIII, HphI, BstNI (partially), AluI, or NsiI, respectively, blunt-ended and digested by BamHI. The hCS promoter 5' deletion fragments were gel-purified and cloned into the HindIII (blunt-ended)/BamHI site of pBLNHCAT3.

hCS453CAT, hCS418CAT, hCS375CAT, hCS113CAT, and hCS84CAT were constructed with specific DNA fragments produced by T4 DNA ligase. The structure of all mutants was confirmed by restriction mapping experiments. The DNA fragments were amplified between these two primers using hCS493CAT as a DNA template. The fragments obtained were digested by HindIII and BamHI, gel-purified, and cloned into the HindIII/BamHI site of pBLNHCAT3.

hCS97CAT was constructed with polymerase chain reaction-amplified fragments obtained with A5 as 5' primer and B2 or B3 as 3' primer, respectively. Amplified fragments were digested by HindIII and BamHI and cloned into the HindIII/BamHI site of pBLNHCAT3.

The oligonucleotides were purchased from Eurogentec (Liége, Belgium) and their sequences are shown as follows.

| Primer | Sense Oligonucleotide | Antisense Oligonucleotide |
|--------|-----------------------|--------------------------|
| A1     | 5'ccccagctTTGGCTGGCTGGCCC' | 3'AGTGACCTATATGGTGGCS' |
| A2     | 5'ccccagctTTCTGTCTGGTGGG' | 3'CTCTCTTGACCGGTCCC' |
| A3     | 5'ccccagctTTAGCACAAGCCGTCAG' | 3'CTGTCTGGTGGGTGG' |
| A4     | 5'ccccagctTTAGGGCCCCCATGCAAAA3' | 3'GCCAAGCTTTCTGGCTGCTGC' |

The DNA fragments were amplified between these two primers using hCS493CAT as a DNA template. The fragments obtained were digested by HindIII and BamHI, gel-purified, and cloned into the HindIII/BamHI site of pBLNHCAT3.

hCS97A NsiI CAT, containing a 5-bp deletion (87--83) was constructed by cleaving hCS97CAT with NsiI and digestion of the protruding ends with Klencow fragment followed by ligation.

The DNA fragments were amplified between these two primers using hCS493CAT as a DNA template. The fragments obtained were digested by HindIII and BamHI, gel-purified, and cloned into the HindIII/BamHI site of pBLNHCAT3.

hCS97A NsiI CAT, containing a 5-bp deletion (87--83) was constructed by cleaving hCS97CAT with NsiI and digestion of the protruding ends with Klencow fragment followed by ligation.

The structure of all mutants was confirmed by restriction mapping and sequencing. All plasmids were prepared by alkaline lysis, purified by centrifugation in CsCl-ethidium bromide gradients, treated with RNase, centrifuged through a cushion of 1 M NaCl, extracted with phenol-chloroform, and finally precipitated with ethanol. The concentration of all plasmid preparations was determined using the absorbance at 260 nm.

Cell Culture Conditions—GC cells were grown in Ham's F-12 medium supplemented with 15% fetal calf serum (Life Technologies, Belgium). One day before transfection, the cells received phenol red-medium supplemented with 15% fetal calf serum pretreated with AG1-

| Primer | Sense Oligonucleotide | Antisense Oligonucleotide |
|--------|-----------------------|--------------------------|
| A1     | 5'ccccagctTTGGCTGGCTGGCCC' | 3'AGTGACCTATATGGTGGCS' |
| A2     | 5'ccccagctTTCTGTCTGGTGGG' | 3'CTCTCTTGACCGGTCCC' |
| A3     | 5'ccccagctTTAGCACAAGCCGTCAG' | 3'CTGTCTGGTGGGTGG' |
| A4     | 5'ccccagctTTAGGGCCCCCATGCAAAA3' | 3'GCCAAGCTTTCTGGCTGCTGC' |

The DNA fragments were amplified between these two primers using hCS493CAT as a DNA template. The fragments obtained were digested by HindIII and BamHI, gel-purified, and cloned into the HindIII/BamHI site of pBLNHCAT3.

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**TABLE I**

**Effect of T3 on various promoter activities**

Induction levels are expressed as the ratio of CAT activities in T3-treated versus untreated cells. Each value is an average of 5 separate transfection experiments performed in triplicate ± S.E. For each promoter, the values obtained from the T3-treated cells are significantly different from those obtained with the untreated cells (p < 0.001).

| Promoter | T3 induction | n |
|----------|--------------|---|
| hCS295CAT | 15.8 ± 1.2 | 3 |
| hCS453CAT | 15.9 ± 1.7 | 4 |
| rGHS30CAT | 5.4 ± 0.6 | 9 |
| hGH496CAT | 0.56 ± 0.04 | 9 |
| TK105VAMP | 1.4 ± 0.5 | 5 |
| RSVVAMP | 1.4 ± 0.1 | 31 |

The DNA fragments were amplified between these two primers using hCS493CAT as a DNA template. The fragments obtained were digested by HindIII and BamHI, gel-purified, and cloned into the HindIII/BamHI site of pBLNHCAT3.

hCS97A NsiI CAT, containing a 5-bp deletion (87--83) was constructed by cleaving hCS97CAT with NsiI and digestion of the protruding ends with Klencow fragment followed by ligation.

The structure of all mutants was confirmed by restriction mapping and sequencing. All plasmids were prepared by alkaline lysis, purified by centrifugation in CsCl-ethidium bromide gradients, treated with RNase, centrifuged through a cushion of 1 M NaCl, extracted with phenol-chloroform, and finally precipitated with ethanol. The concentration of all plasmid preparations was determined using the absorbance at 260 nm.

**FIG. 1.** T3 induction of CAT expression of 5' deletion mutants of the hCS-B promoter linked to the CAT reporter gene. Induction levels are expressed as the ratio of CAT activities in T3-treated versus untreated cells. All data are averages ± S.E. of at least six independent cell cultures. The basal activities of the various constructs, expressed versus that of the hCS494CAT construct, are given below for information. The activities of the various constructs down to hCS160CAT do not differ significantly from that of the hCS494CAT construct. Further deletions (hCS129CAT, hCS117CAT, hCS98CAT) decrease the basal activity about 3-fold, and the shortest construct, hCS84CAT, has a basal activity about 7-fold lower than hCS494CAT. The CAT activity measured for mock-transfected cells is less than 1% of that obtained for hCS494CAT activity. The binding sites for the ubiquitous NF1 and SP1 factors as for the pituitary-specific GHFl factor are indicated by boxes (16). The distal GHFl binding site, only seen with high extract concentration, is not shown.
X8 resin (23) and with activated charcoal to eliminate thyroid and steroid hormones. Addition of [125I]T3 to the serum before hormone depletion indicated that the maximal level of T3 in the final media was below $2 \times 10^{-7}$ M.

Transfection by Electroporation—GC cells were harvested with trypsin/EDTA and resuspended in phenol red-free Ham’s F-12 medium at the concentration of 3 x 10⁵ cells/ml. Supernoded plasmid DNA (10 or 30 μg for hCS2289CAT and its 5’ deletion mutants, 5 μg for the TKCAT constructs, rGH530CAT and RSVamp) were mixed with 24 x 10⁶ cells and exposed to a single 1500-μF and 250-V/4 mm discharge delivered by a “Cellject” electroporator (EquiBio Liege, Belgium). Cells were rapidly transferred to phenol red-free Ham’s F-12 medium containing steroid- and thyroid hormone-depleted calf serum. Half of the transected cells were incubated with 10 nM T3, and the other half received the same amount of ethanol/NaOH as used to dissolve T3. 40 h after electroporation, the cells were harvested by scraping, washed in phosphate-buffered saline, resuspended in 100 μl of 250 mM Tris-HCl, pH 7.6, for the CAT assay and in 100 μl of 50 mM sodium phosphate buffer, pH 6.0, for the β-lactamase assay and then frozen at -70°C. The cells were disrupted by three cycles of freezing and thawing, and the samples were centrifuged at 12,000 × g in an Eppendorf centrifuge. CAT and β-lactamase assays were performed as described previously (24).

Nuclear Extract Preparation from Infected HeLa Cells—Recombinant vaccinia virus expressing the chicken c-erb A protein (12) and the wild type (wt) vaccinia virus were kindly provided by Dr. Stunnenberg (EMBL, Heidelberg, Federal Republic of Germany). For infection, we used human cervical carcinoma (HeLa S3) cells, grown in continuous suspension culture in Joklik modified Eagle’s medium supplemented with 10% fetal calf serum. 5 x 10⁵ HeLa cells were infected with recombinant or wt vaccinia virus and nuclear extracts were prepared as described by Dignam et al. (25). Briefly, 16 h after infection, the cells were harvested, washed with phosphate-buffered saline, and suspended in 5–10 packed cell pellet volumes of buffer A (10 mM Hepes, pH 7.9, 5 mM MgCl₂, 10 mM NaCl, 1 mM DTT; 0.1 mM phenylmethylsulfonyl fluoride). The cells were collected by centrifugation, suspended again in 5 packed cell pellet volumes of buffer A, and lysed by 10–20 strokes of Dounce homogenizer (B-type pestle). The homogenate was checked microscopically for cell lysis and centrifuged for 5 min at 2000 × g. The crude nucleus pellet was washed with 2.5 volumes of buffer A to remove residual cytoplasmic material and resuspended in 2 volumes of buffer C (20 mM Hepes, pH 7.9, 5 mM MgCl₂, 0.1 mM EDTA, 1% glyceral, 1 mM DTT; 0.1 mM phenylmethylsulfonyl fluoride). The final NaCl concentration was adjusted to 420 mM and incubated for 45 min on ice with stirring. The nuclear extract was cleared by centrifuging for 30 min at 25,000 × g. The supernatant, called the “nuclear extract,” was passed onto a Centricon 10 cartridge (Amicon) to lower NaCl concentration to about 200 mM, and stored at -70°C. The protein concentration was 4.5 mg/ml, and 20 μg of protein were obtained from 5 x 10⁶ infected cells.

DNete I Footprinting—DNase footprinting experiments were performed as described by Sapp et al. (12). End-labeled DNA (1–5 ng; specific activity about 10⁶ cpm/ng) was incubated with cell extract (0 or 10 μl) and 6 μg of poly(dI-dC) in a final volume of 60 μl of DNA binding buffer (10 mM Hepes, pH 7.8, 2 mM MgCl₂, 1 mM EDTA, 5% glycerol, 0.1 mg/ml bovine serum albumin). The final salt concentration was adjusted to 40 mM by addition of KCl. After a 30-min incubation at 30°C, 2 μl of an appropriate dilution of DNase I (0.3–3 units; Boehringer Mannheim) were added and digestion allowed to proceed for 100 s at 20°C. The reaction was stopped, and fragments were analyzed on 5% polyacrylamide, 5 M urea sequencing gels.

Thyroid Hormone Binding Assays—Thyroid hormone receptor content and affinity were assessed as described previously (20) by incubating 1 μl of nuclear extract with various concentrations (0.01–0.06 nm) of [125I]T3 (3 μCi/μg; Amersham Corp.) in 20 mM N-tris(hydroxymethyl)methylglycine, 1 mM EDTA, 2 mM DTT, 20% glycerol, 50 mM NaCl, 0.1 mg/ml bovine serum albumin. A parallel series of tubes were supplemented with 0.5 μM unlabelled T3 for determining nonspecific binding. After 4 h at room temperature, 0.3 ml of an ice-cold solution of hydroxylapatite (15 g/100 ml in 20 mM Tris-HCl, 10 mM KH₂PO₄, pH 7.2) was added to the tubes, and the mixture was further incubated for 10 min on ice. After centrifugation (1000 × g for 2 min), the hydroxylapatite pellet was washed three times with 10 mM Tris-HCl, 5 mM NaCl, 1.5 mM EDTA, 0.5% Triton X-100, pH 7.2, and the residual radioactivity was determined.

Statistical Analysis—Statistical comparisons of means were performed by two-way analysis of variance.

RESULTS

The Activity of the hCS-B Promoter Is Stimulated by T3 in GC Cells—To analyze the T3 regulation of the hCS-B promoter activity, we used the plasmids hCS2289CAT and hCS493CAT where the proximal 2289 and 493 bp of the hCS-B promoter are inserted upstream from the CAT reporter gene. These constructs were introduced into GC cells by electroporation. Transfected cells were immediately incubated with or without 10 nM T3, and 40 h later, the CAT activity was determined in the transfected cell extracts. As the treated and untreated cells originated from the same pool of transfected cells, the observed differences between control and treated cells could only be attributed to the T3 treatment and not to variations in transfection efficiency.

Table I summarizes the results obtained for at least three separate transfection experiments. hCS2289CAT and hCS493CAT are equally stimulated by T3 (about 16-fold). This stimulation is much stronger than that obtained with the 530-bp rat GH promoter (rGH530CAT) whose activity is
well known to be stimulated by T3 (18). On the other hand, the hGH promoter activity in hGH496CAT is significantly ($p < 0.001$) inhibited by T3 as shown previously (13). The negative controls TK105CAT and RSVamp show a slight 1.4-fold nonspecific stimulation by T3.

5' Deletion Analysis of the hCS-B Gene Promoter.—To locate the 5' boundary of the hCS-B thyroid response unit (TRU), a set of 5' deletions were made in the hCS-B promoter. These mutated promoters were fused to the CAT reporter gene and introduced into GC cells and tested for their T3 response. Fig. 1 presents the degree of T3 induction obtained for each mutant.

A small (approximatively 2-fold) diminution in T3 induction is observed between coordinates −493 and −375. Plasmids with promoter endpoints −375, −295, −212, −171, −160, −128, −113, and −97 were equally responsive to T3 (about 7-fold stimulation). A major decrease in the T3 response occurred when the sequence −97 to −84 was removed (stimulation dropped from 7- to 0.6-fold). The observed lack of stimulation of the 83-bp promoter activity is not due to an insufficient basal expression of hCS83CAT, since the latter is at least 10-fold higher than the background level (data not shown).

These results demonstrate that two regions are involved in the T3 response: a distal region located between coordinates −493 and −373 and a proximal region with its 5' boundary between coordinates −97 and −83. In fact, this later region is part of the pituitary-specific GHFl factor binding site (−95/−68) (16). This suggests either that the T3 receptor interacts with the DNA at the level of the GHFl binding site or that the GHFl factor is required for T3 induction of the hCS promoter activity.

3' Deletion Analysis of the hCS-B Gene Promoter.—To locate the proximal TRU 3' boundary, we cloned different parts of the hCS-B promoter upstream from the TK promoter. Fig. 2 shows that the hCS −97 to −31 region is able to confer T3 stimulation to the otherwise unresponsive TK promoter (see [hCS−97/−31]TKCAT). A 3' deletion of the −31 to −46 sequence ([hCS−97/−47]TKCAT) causes complete loss of stimulation, thus indicating that the TRU 3' boundary lies between these coordinates. Of course, this lack of stimulation is also observed with the [hCS−92/−72]TKCAT construct that contains only the GHFl binding site.

In conclusion, by means of 5' and 3' deletions of the hCS promoter (Figs. 1 and 2), we have defined the minimal DNA elements A and B. DNA fragment labeling and DNase I reaction were carried out as described under "Experimental Procedures." Lane 1, no extract added; Lane 2, 45 μg of the c-erhA extract; Lane 3, 45 μg of the wet extract. The markers (M) are pBLCAT2 digested by HpaII, TsaI, and Sau3AI. Open boxes alongside the autoradiographs indicate the DNase I protected sites with the coordinates of their respective 5' and 3' borders. The arrow shows a DNase I-hypersensitive site (HS). c, sequence of the thyroid receptor (TR) binding site. The lines indicate the protected regions on the sense strand (B) and on the antisense strand (A). d, location of the TR binding site in the TRU.
sequence required for T3 induction of the hCS-B promoter. This thyroid response unit (TRU) spans the 67 bp extending from coordinate -97 to -31.

**Interaction of the T3 Receptor with the hCS-B Promoter**

To precisely identify the DNA sequences that interact with the thyroid receptor in the TRU, we performed DNase I footprinting.

For this purpose, the chicken thyroid hormone receptor-α protein was overproduced using a vaccinia virus expression system. Crude nuclear extracts were prepared from c-erbA-vaccinia-infected HeLa cells (c-erbA extract) and, as a control, from HeLa cells infected with wt vaccinia virus (wt extract). Scatchard analysis of T3 binding to these two nuclear extracts demonstrated approximately 40-fold overexpression of thyroid receptor in c-erbA extract as compared with wt extract (data not shown).

DNase I footprinting (Fig. 3) carried out with end-labeled fragment A (labeled antisense strand) revealed that the c-erbA extract protects the hCS-B promoter region between coordinates -67 and -41 against DNase I digestion (gel A, lane 2). Simultaneously a DNase I-hypersensitive site (HS) appeared at the -67 border. This protection is not observed with the wt extract (gel A, lane 3). In contrast, protection of the ubiquitous SP1 binding site (-141/-117) is detected with both extracts, demonstrating that the extracts are of equal quality.

The presence of this thyroid receptor binding site is confirmed by DNase I footprinting performed with fragment B (labeled sense strand). In this case, protection also extends over a length of about 25 nucleotides (from -67 to -42) in the presence of c-erbA extract (gel B, lane 2) but not in the presence of wt extract (gel B, lane 3).

The sequence of this thyroid receptor binding site is indicated in Fig. 3, together with its location in the TRU.

We also performed footprinting experiments on the (-500 to -290) region of the hCS promoter, since T3 induction level is halved when this region is deleted (see Fig. 2). No clear T3 receptor footprint could be detected in this region, suggesting that binding of the T3 receptor to site(s) in this region is weaker than binding to the proximal TRU. Additional footprinting experiments in T3 receptor will probably be required to detect the sequence(s) interacting with the T3 receptor in this region.

**GHF1 Is Necessary for T3 Induction of the hCS-Promoter**

The observation in Fig. 1 that a 5′ deletion mutant with an endpoint at -83 shows no T3 induction is unexpected, considering the fact that this mutant contains the entire T3 receptor binding site (−67 to −41) as defined by footprinting experiments (Fig. 3). This suggests that the T3 receptor binding site alone is not sufficient for T3 induction. Accessory elements such as the GHF1 binding site (−95/−68) also seem to be required.

To further test this hypothesis, we destroyed the GHF1 binding site in the hCS97CAT construct by introducing a 5′ deletion in the GHF1 consensus motif (TTATGNA) (18, 27). In this deleted construct (hCS97ΔNsiICAT), the sequence (−92) GCCATGATAA (−81) has been changed to CCCAAA. An analogous deletion has been shown to abolish GHF1 binding at its proximal site in the equivalent hGHANsiI mutant (16). Our mutant was introduced into GC cells, and its T3 response was compared with the response observed with the undeleted construct.

Table II shows a total loss of T3 stimulation for hCS97ΔNsiICAT. This again demonstrates the essential role of the GHF1 binding site in the T3 induction of the hCS-B promoter.

**Comparison between the hGH-N and hCS-B Promoters**

As shown in Table I, the proximal 500 bp of the hGH-N and hCS-B gene promoters are regulated in opposite ways by T3. This is unexpected as the overall similarity of the two promoters is 94% (Fig. 4). The similarity is, however, considerably lower (80%) at the level of the sequence which interacts in hCS-B with the thyroid receptor: 5′ (out of 25) positions differ between these two sequences (see Fig. 4).

To determine the influence of these differences on T3 receptor binding, we subjected the hGH-N promoter to DNase I footprinting (Fig. 5). As for the hCS promoter, both the c-erbA (lane 3) and wt (lane 3) extracts protect the ubiquitous SP1 binding sites against DNase I digestion. In contrast, only the c-erbA extract protects the −70 to −48 region of the hGH-N promoter. Compared with hCS, the location of hGH protection is thus slightly shifted upstream. In addition, this protection is slightly weaker and there is no DNase I-hypersensitive site at the protected region’s 5′ boundary as in hCS-B.

**DISCUSSION**

By means of 5′ and 3′ deletion mutants of the hCS-B promoter, we have located the minimal sequence required for T3 induction of hCS-B promoter activity. The sequence spans 67 bp, from coordinate −97 to −31 and contains a thyroid hormone receptor binding site (−67/−41) and a pituitary-specific GHF1 binding site (−56/−68). Neither site taken alone is able to confer thyroid responsiveness. Both are also found in the hGH-N promoter, which is nevertheless down-regulated by T3.

**Analysis of the Thyroid Receptor Binding Element**

In order to compare the hCS TRE with other reported TRES, we divided it into two domains, A and B, and analyzed the two domains independently (Fig. 6). Some TRES contain se-
T3 Stimulation of hCS-B Promoter Activity

![Diagram](image-url)

**Fig. 5.** DNase I footprinting analysis of specific c-erbA binding to the hGH-N gene promoter. **a,** schematic representation of the $^{32}$P-labeled DNA used in the footprinting experiments. The binding site for the ubiquitous SP1 factor is indicated by a box (20, sequences very similar to the 5′ portion of the hCS TBE (Domain A). This domain contains the motif TGGGGTCA, closely resembling the half-palindrome TcaGGTCA described by Glass et al. (1) and the consensus T3 receptor binding half-site aGGTC/aA proposed by Brent et al. (7). At this first category belongs the TRE of the chicken lysosyme which contains two such motifs disposed in palindromic structure (28).

Other TRES contain sequences similar to the 3′ portion of the hCS TBE (Domain B) (e.g. human myosin heavy chain TRES (8)). This region is characterized by a high purine content (13 purines out of 14 nucleotides). An analogous purine-rich motif GGAGGACAG, found in the 3′ portion of the rat malic enzyme (rME) and α-myosin heavy chain genes' TRES, has been suggested to be important in determining the affinity of the thyroid receptor for the TRE (9).

Finally, a third category of TRES exhibits homology with the whole hCS TBE and thus contains both A and B domains. Among these are the rME TRE (9) and the rat prolactin sequence (−1559/−1531) known to be essential to T3 stimulation of PRL expression (29).

Sequence analysis of the hCS TBE reveals 2 interesting features: first, this TBE contain but one copy of the hexameric consensus sequence proposed by Brent et al. (7) (Fig. 6), although these authors have shown that at least two such motifs are required for T3 induction. We suggest that the purine-rich region might compensate for the lack of a repeat, thus playing a major role in thyroid receptor binding. Second, the hCS TBE does not contain any striking palindrome or repeat. This is surprising in view of the suggestion that thyroid receptors, like their steroid counterparts (30), interact as dimers with palindromic sequences (1). Another protein (present in HeLa cells used to overproduce the TR receptor) might interact with domain B to stabilize interaction between the monomeric T3 receptor and domain A. Enhancement of thyroid receptor binding by accessory nuclear factors has already been demonstrated (31, 32) as the ability of the thyroid receptor to form heterodimers with other receptors (33, 34).

**Comparison of the hCS-B and hGH-N Promoters—**Our results confirm T3 inhibition of the 496-bp hGH promoter's activity, presented in previous studies (13). This regulation contrasts with the marked stimulatory effect of T3 on the 94%-similar hCS promoter. Sequence comparisons show,
however, that similarity between the two promoters is clearly lower in the region of the hCS TBE, there being five differing positions in this 25-bp sequence (80% similarity). DNase I footprinting has shown that the thyroid receptor is able to bind the hGH promoter, but the footprint’s position is slightly shifted upstream as compared with the hCS promoter (−70/−48 for hGH-N and −67 to −41 for hCS) (see Fig. 4). Furthermore for the same protein concentration, protection is less complete for hGH than for hCS, suggesting that the thyroid receptor’s affinity for the hGH sequence is lower. We thus propose that the mutations in hGH sequence, although not preclusive of thyroid receptor binding, reduce the affinity of the thyroid receptor for this site. Quantitative studies will be required to confirm this view.

From these observations, we can offer two possible explanations as to why the hGH-N promoter is not stimulated by T3. The first is that the T3 receptor’s affinity for the hGH site might not be sufficient to allow activation. The second is that the protected region’s shift in hGH leads to overlapping of the GHFl and thyroid receptor binding sites, whereas these sites are adjacent in hCS-B (Fig. 4). In this case, we suggest that synergism between the two factors is hindered, thus preventing stimulation of hGH-N promoter activity. To test this hypothesis, it will be interesting to perform footprinting experiments in the presence of both factors. A diminution of GHFl binding would explain the down-regulation of hGH promoter activity and loss binding of the T3 receptor would explain the lack of stimulation of hGH promoter activity. Unfortunately, our extract is insufficiently enriched in T3 receptor and does not allow such an experiment. We are currently attempting to increase the overexpression of the thyroid receptor to make such footprinting possible.

**The GHFl Binding Site Is Necessary for the T3 Response**—The GHFl factor’s essential role in the hCS-B promoter’s T3 response has been established here by means of two deletions: a 14-bp deletion of coordinates −97 through −84, removing a major part of the GHFl binding site, and a shorter, 5-bp deletion (−87/−83) at the center of the GHFl consensus motif. Both deletions, although leaving the thyroid receptor binding site intact, cause complete loss of T3 stimulation (see Fig. 1 and Table II). GHFl alone is nevertheless insufficient to promote T3 stimulation, as proved by the lack of stimulation of the [hCS-94/−72]TKCAT construct, containing only the GHFl binding site, and of the longer [hCS-97/−47] TKCAT construct.

This study thus points to the importance of functional interactions between the thyroid receptor and other transcriptional factors in inducing an effective thyroid hormone response. Ye et al. (35) have also suggested such interactions. They have shown that both a distal thyroid response element and a cell-specific basal element are required for efficient T3-regulated expression of the rat growth hormone gene. To date, however, we know of no other data pointing to a functional interdependence of the thyroid receptor and unrelated transcriptional factors.

In contrast, functional interactions between steroid receptors and other essential factors are well documented. For instance, mutational analysis of the MMTV promoter has revealed that inducibility by glucocorticoids is strongly dependent on the integrity of an NF1 motif (36). Later experiments artificially combining glucocorticoid-responsive elements with various transcription factor binding sites have shown that not only NF1 but a whole battery of other well-characterized factors are able to act in combination with the glucocorticoid receptor to activate an adjacent promoter (37, 38). In this case, the observed cooperativity is mediated by protein-protein interaction and does not depend on cooperative binding to DNA. Such interactions seem especially important when the binding site for the receptor is not very strong (38) and/or is not in close proximity to the TATA box (39).

In the hCS-B promoter, the GHFl and thyroid receptor binding sites are in fact contiguous. Such proximity is also found for the glucocorticoid-responsive elements, which are often tightly clustered with other regulatory sequences (40). As the placent cells do not contain GHFl factor, it will be interesting to investigate whether another trans-acting factor (e.g. SP1) can interact with the thyroid receptor and thus allow T3 stimulation of the hCS-B promoter activity. Experiments are currently underway to examine this hypothesis.

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**REFERENCES**

1. Glass, C. K., Holloway, J. M., Devary, O. L., and Rosenfeld, M. G. (1988) Cell 54, 313–323
2. Samuels, H. H., Forman, B. M., Horowitz, Z. D., and Ye, Z-S. (1989) Annu. Rev. Physiol. 51, 623–639
3. Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghydhael, J., Leutz, A., Beug, H., and Vennstrom, B. (1986) Nature 324, 635–640
4. Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruiol, D. J., and Evans, R. M. (1986) Nature 324, 641–646
5. Forman, B. M., and Samuels, H. H. (1990) Mol. Endocrinol. 4, 1295–1301
6. Evans, R. M. (1988) Science 240, 889–895
7. Brent, G. A., Harney, J. W., Shen, Y., Warner, L. M., Moore, D. D., and Larsen, P. R. (1989) Mol. Endocrinol. 3, 1966–2004
8. Fink, I. L., and Morkin, E. (1990) J. Biol. Chem. 265, 11233–11237
9. Petty, K. J., Desvergne, B., Mitsuhashi, T., and Nikodem, V. M. (1990) J. Biol. Chem. 265, 7395–7400
10. Norman, M. F., Lavin, T. N., Baxter, J. D., and West, B. L. (1989) J. Biol. Chem. 264, 12063–12073
11. Sap, J., Munoz, A., Schmittelunghen, B., and Vennstrom, B. (1989) Nature 340, 242–244
12. Sap, J., Magistris, L., Stunnenberg, H., and Vennstrom, B. (1990) EMBO J. 9, 887–896
13. Morin, A., Louette, J., Voz, M. L., Texier-Vidal, A., Belayev, A., and Martial, J. A. (1990) Mol. Cell. Endocrinol. 71, 261–267
14. Cattini, P. A., and Eberhardt, N. L. (1987) Nucleic Acids Res. 15, 1297–1309
15. Nachtigal, M. W., Nickel, B. E., Klassen, M. E., Zhang, W., Eberhardt, N. L., and Cattini, P. A. (1989) Nucleic Acids Res. 17, 4327–4337
16. Lemaigre, F. P., Peers, B., Lafontaine, D. A., Mathy-Hartert, M., Rousseau, G. G., Belayev, A., and Martial, J. A. (1989) DNA (N.Y.) 8, 149–159
17. Nickel, B. E., Kardami, E., and Cattini, P. A. (1990) Biochem. J. 267, 653–658
18. Flug, F., Copp, R. P., Casanova, J., Horowitz, Z. D., Janlocko, L., Plotnick, M., and Samuels, H. H. (1987) J. Biol. Chem. 262, 6373–6382
19. Luckow, B., and Schütz, G. (1987) Nucleic Acids Res. 15, 5490
20. Lefevre, C., Imagawa, M., Dana, S., Grindlay, J., Bodner, M., and Karin, M. (1987) EMBO J. 6, 971–981
21. Muller, M., Belfroid, E., Lambert, C., Belayev, A., and Martial, J. A. (1988) Arch. Int. Physiol. Biochim. 94, B35
22. Laimins, L. A., Khoury, G., Gorman, C., Howard, B., and Gruss, P. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6453–6457
23. Samuels, H. S., Stunnenberg, H., and Casanova, J. (1979) Endocrinology 105, 80–85
24. Peers, B., Voz, M. L., Monget, P., Mathy-Hartert, M., Belayev, A., and Martial, J. A. (1986) Mol. Cell. Biol. 10, 4690–4700
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25. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475-1489
26. Barlow, J. W., Voz, M. L. J., Eliard, P. H., Mathy-Hartert, M., De Nayer, P., Economides, I. V., Belayew, A., Martial, J. A., and Rousseau, G. G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9021-9025
27. Nelson, C., Albert, V. R., Elsholtz, H. P., Lu L. I.-W., and Rosenfeld, M. G. (1988) Science 239, 1400-1405
28. Baniahmad, A., Steiner, C., Kohne, A. C., and Renkawitz, R. (1990) Cell 61, 505-514
29. Day, R. N., and Maurer, R. A. (1989) Mol. Endocrinol. 3, 931-938
30. Scheiderreit, C., Westphal, H. M., Carlson, C., Bosshard, H., and Bento, M. (1986) DNA (N. Y.) 5, 383-391
31. Murray, M. B., and Towle, H. C. (1989) Mol. Endocrinol. 3, 1434-1442
32. Burnside, J., Darling, D. S., and Chin, W. W. (1990) J. Biol. Chem. 265, 2500-2504
33. Forman, B. M., Yang, C.-R., Au, M., Casanova, J., Ghysdael, J., and Samuels, H. H. (1989) Mol. Endocrinol. 3, 1610-1626
34. Glass, C. K., Lipkin, S. M., Devary, O. V., and Rosenfeld, M. G. (1989) Cell 59, 679-708
35. Ye, Z. S., Forman, B. M., Aranda, A., Pascual, A., Park, H. Y., Casanova, J., and Samuels, H. H. (1988) J. Biol. Chem. 263, 7821-7829
36. Miksicek, R., Borgmeyer, U., and Nowock, J. (1987) EMBO J. 6, 1355-1360
37. Schule, R., Muller, M., Otsuka-Murakami, H., and Renkawitz, R. (1988) Nature 332, 87-90
38. Schule, R., Muller, M., Kaltzschmidt, C., and Renkawitz, R. (1988) Science 242, 1418-1420
39. Strahle, U., Schmid, W., and Schutz, G. (1988) EMBO J. 7, 3389-3395
40. Steiner, C., Muller, M., Baniahmad, A., and Renkawitz, R. (1987) Nucleic Acids Res. 15, 4163-4178
41. Lemaigre, F. P., Courtois, S. J., Lafontaine, D. A., and Rousseau, G. G. (1989) Eur. J. Biochem. 181, 555-561
42. Courtois, S. J., Lafontaine, D. A., Lemaigre, F. P., Durviaux, S. M., Rousseau, G. G. (1990) Nucleic Acids Res. 18, 57-64
43. Glass, C. K., Franco, R., Weinberger, C., Albert, V., Evans, R. M., and Rosenfeld, M. G. (1987) Nature 329, 738-741
44. Darling, D. S., Burnside, J., and Chin, W. W. (1989) Mol. Endocrinol. 3, 1359-1368
45. Burnside, J., Darling, D. S., Carr, F. E., and Chin, W. W. (1989) J. Biol. Chem. 264, 6886-6891
46. Izumo, S., and Mahdavi, V. (1998) Nature 334, 539-542