

Thyroid hormone receptors bind to defined regions of the growth hormone and placental lactogen genes

(chorionic somatomammotropin/DNA binding/glucocorticoids/somatotropin)

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ABSTRACT The intracellular receptor for thyroid hormone is a protein found in chromatin. Since thyroid hormone stimulates transcription of the growth hormone gene through an unknown mechanism, the hypothesis that the thyroid hormone-receptor complex interacts with defined regions of this gene has been investigated in a cell-free system. Nuclear extracts from human lymphoblastoid IM-9 cells containing thyroid hormone receptors were incubated with L-3,5,3'-tri[¹²⁵I]iodothyronine and calf thymus DNA-cellulose. Restriction fragments of the human growth hormone gene were added to determine their ability to inhibit labeled receptor binding to DNA-cellulose. These fragments encompassed nucleotide sequences from about three kilobase pairs upstream to about four kilobase pairs downstream from the transcription initiation site. The thyroid hormone-receptor complex bound preferentially to the 5'-flanking sequences of the growth hormone gene in a region between nucleotide coordinates -290 and -129. The receptor also bound to an analogous promoter region in the human placental lactogen gene, which has 92% nucleotide sequence homology with the growth hormone gene. These binding regions appear to be distinct from those that are recognized by the receptor for glucocorticoids, which stimulate growth hormone gene expression synergistically with thyroid hormone. The presence of thyroid hormone was required for binding of its receptor to the growth hormone gene promoter, suggesting that thyroid hormone renders the receptor capable of recognizing specific gene regions.

L-3,5,3'-Triiodothyronine (T3), the active form of thyroid hormone, modulates the expression of a number of genes (1, 2), and this is ascribed to the association of T3 with receptors localized in chromatin (3). In rat pituitary tumor cells the concentration of growth hormone (GH) mRNA increases in response to physiological concentrations of T3 (refs. 4 and 5). Although additional mechanisms have not been ruled out, this response reflects an increased rate of transcription of the GH gene (6, 7), presumably through a direct effect of the receptor (8). Moreover, T3 acts synergistically with glucocorticoid hormones in stimulating GH gene transcription (6, 9). Binding regions for the rat (10, 11) and human (12) glucocorticoid receptors have been identified in the human GH gene upstream and downstream from the transcription initiation site (cap site). Homologous binding regions have also been detected (12) in the human placental lactogen (chorionic somatomammotropin, CS) gene, which has a 92% overall nucleotide sequence homology with the human GH gene (13). In contrast, the question of specific recognition sites for thyroid hormone receptors on any DNA has re-

mained open. We have now searched for such sites in the human GH and CS genes (12) using the human thyroid hormone receptor in a cell-free system. This system contained [¹²⁵I]T3-labeled nuclear extracts from cultured human lymphoblastoid cells of the IM-9 line and cloned fragments from the genes of interest.

MATERIALS AND METHODS

Preparation of Receptor-Containing Nuclear Extracts. IM-9 cells were grown as suspension cultures at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum and 50 units of penicillin and of streptomycin per ml (all from GIBCO). Cells in the stationary phase of growth ($1-2 \times 10^6$ cells per ml) were harvested by centrifugation and washed twice in serum-free medium. A nuclear pellet was obtained from cells lysed in a buffer containing 0.25 M sucrose, 1.1 mM MgCl₂, 20 mM Tris-HCl, and 0.5% Triton X-100 (pH 7.85). Thyroid hormone receptors were extracted with 20 mM Tris-HCl, 0.4 M KCl, 2 mM EDTA, 1.1 mM MgCl₂, and 5 mM dithiothreitol (pH 7.85) followed by a 1- to 2-s burst with a Branson sonicator and ultracentrifugation for 25 min at $100,000 \times g$. The resulting supernatant (nuclear extract), which contained about 5 mg of protein per ml, was stored at -80°C until use (within 1 month). Thyroid hormone receptor content and affinity were assessed by incubating 38.5 μ l of extract with various concentrations (0.01-0.4 nM) of [¹²⁵I]T3 (3 mCi/ μ g; 1 Ci = 3.7×10^{10} becquerels; Amersham) in 20 mM N-tris[hydroxymethyl]methylglycine, 1 mM EDTA, 2 mM dithiothreitol, and 20% glycerol (pH 7.4) (TEDG buffer) in a final volume of 0.3 ml. A parallel series of tubes were supplemented with 0.3 μ M unlabeled T3 for determination of nonspecific binding. After 4 hr at room temperature, 0.3 ml of an ice-cold solution of hydroxyapatite (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 ($1000 \times g$ for 2 min), the hydroxyapatite pellet was washed three times with 10 mM Tris-HCl, 5 mM NaH₂PO₄, 1.5 mM EDTA, and 0.5% Triton X-100 (pH 7.2), and the residual radioactivity was determined.

DNA-Binding Assay for the Thyroid Hormone Receptor. The cloning of the GH-N and CS-B genes and the purification and preparation of blunt-ended fragments have been described (12). The DNA-cellulose-binding assay for thyroid hormone receptors was designed on the basis of the competition assay used to study the binding of unpurified gluco-

Abbreviations: bp, base pair(s); CS, chorionic somatomammotropin; GH, growth hormone; kb, kilobase(s); T3, L-3,5,3'-triiodothyronine. ‡To whom reprint requests should be addressed at: Université Catholique de Louvain-International Institute of Cellular and Molecular Pathology, 75 Avenue Hippocrate, B-1200 Brussels, Belgium.

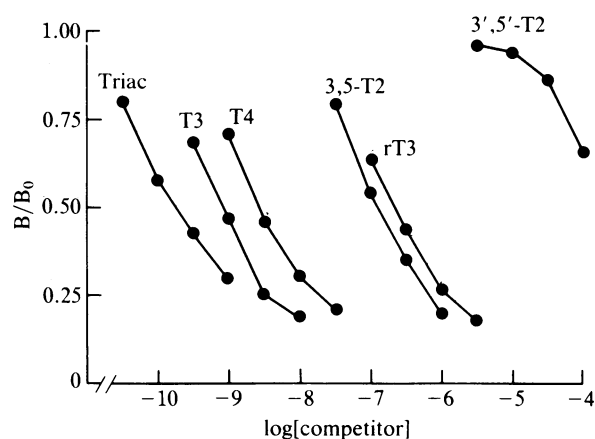


FIG. 1. Competition of thyroid hormone analogues for [125 I]T3 binding to the human nuclear receptor. Nuclear extracts were prepared and incubated with [125 I]T3 and the indicated concentrations of unlabeled analogues: 3,5,3'-triiodothyroacetic acid (Triac), L-thyroxine (T4), 3,5-diiodothyronine (3,5-T2), 3',5',3-triiodothyronine (rT3), 3',5'-diiodothyronine (3',5'-T2). After 4 hr at room temperature, the amount of receptor-bound [125 I]T3 was determined by the hydroxyapatite assay. Results are expressed as the ratio of T3 bound specifically in the presence (B) to that bound in the absence (B_0) of competitor. Results shown are means for three or more experiments, with SEM < 0.05 and a B_0 value of 5250–9500 dpm.

corticoid receptor to DNA (12). Double-stranded calf thymus DNA-cellulose (12) was washed three times with 3 vol of TEDG buffer and resuspended in 40 vol of this buffer. Incubations (0.3 ml) contained the equivalent of 1.25 μ l of packed DNA-cellulose, 50 μ l of nuclear extract (5.4 fmol of T3-binding sites), 0.1 nM [125 I]T3, and up to 500 ng of competing DNA fragment. Incubations (final KCl concentrations, 66 mM) were at room temperature with constant

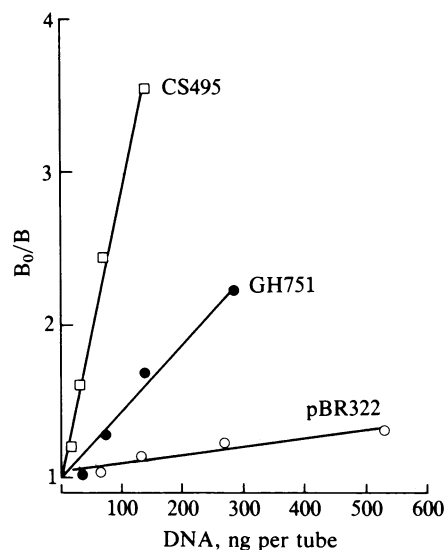


FIG. 2. Thyroid hormone receptor binding to cloned DNA fragments. Increasing concentrations of DNA fragments were incubated with DNA-cellulose, nuclear extract, and [125 I]T3. After equilibrium was attained the DNA-cellulose was washed and the residual radioactivity associated with the DNA-cellulose pellet was determined. The competition curves generated (all points are from duplicate incubations) were linearized by regression after expressing the ratio of radioactivity bound to DNA-cellulose in the absence (B_0) to that bound in the presence (B) of competing DNA, as a function of competing DNA concentration. CS495: fragment *EcoRI* (–494)–*BamHI* (+2) of the CS gene; GH751: fragment *Sac I* (–750)–*BamHI* (+2) of the GH gene; pBR322: mixture of 506- and 516-bp-long fragments of pBR322 after digestion with *HinfI*.

end-over-end rotation. After 4 hr the tubes were placed on ice and the DNA-cellulose pellet was sedimented in a Microfuge

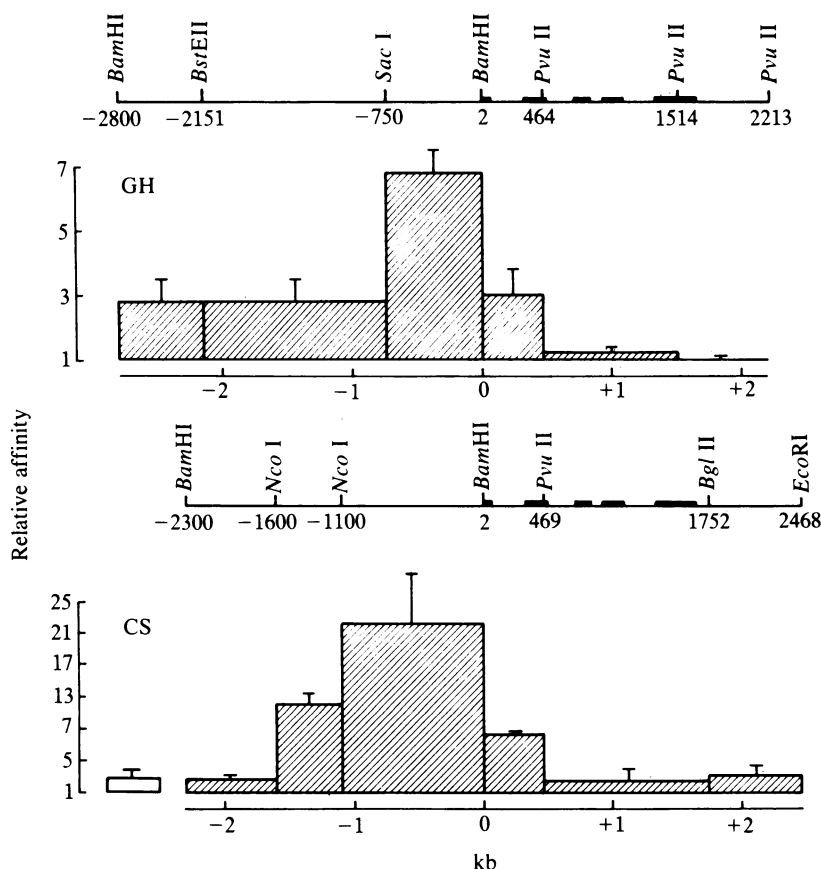


FIG. 3. Relative affinity of the thyroid hormone receptor for DNA along the GH and CS genes. The ordinate gives the slope of competition curves (three to five data points) obtained with the corresponding fragment (see Fig. 2). The results shown are means $\pm$ SEM for at least three experiments. The thickened line indicates exons. Results obtained with the mouse mammary tumor virus DNA fragment LTR451 (open box at the bottom left) are shown for comparison. Not shown are two 3'-flanking GH gene fragments *Pvu II* (+2213)–*Pvu II* (+2613) and *Pvu II* (+2613)–*BamHI* (+3913), which yielded slopes of 1.0 and 2.0 $\pm$ 0.2, respectively.

for 30 s, washed three times with ice-cold TEDG buffer containing 50 mM NaCl, and assayed for radioactivity. Nonspecific binding was determined from identical incubations except for the addition of either 0.3 μ M unlabeled T3 or 3600 ng of salmon sperm DNA. Radioactivity bound to DNA-cellulose was the same in both cases.

RESULTS AND DISCUSSION

Characterization of the Thyroid Hormone-Binding Sites in IM-9 Cell Nuclei. The receptor-DNA-binding assay is based on the ability of DNA fragments from the genes studied to interfere *in vitro* with the binding of thyroid hormone receptors labeled with [125 I]T3 to calf thymus DNA immobilized on cellulose. The receptor preparation was a nuclear extract from IM-9 cells. When whole cells were incubated at 37°C for 2 hr with different concentrations (0.01–0.4 nM) of [125 I]T3, analysis of the saturable T3 binding in the nuclear fraction showed a single class of sites with an affinity and capacity identical to those found in circulating human lymphocytes (14) ($K_d = 34 \pm 6$ pM, 346 ± 30 sites per cell, 38 ± 3 fmol/mg of DNA, mean $\pm$ SEM for 4 experiments). Cell-free binding and characterization of these sites solubilized from nuclei provided further evidence that they correspond to thyroid hormone receptors. The linear Scatchard (15) plot (not shown) was compatible with a homogeneous class of noninteracting sites (K_d at 25°C = 0.14 ± 0.01 nM; site concentration = 22.4 ± 2.5 fmol/mg of protein, mean $\pm$ SEM for 14 experiments). The relative potency with which unlabeled thyroid hormone analogues competed with [125 I]T3 for binding (Fig. 1) was as described for other mammalian thyroid hormone receptors (2).

DNA Binding of the Thyroid Hormone Receptor. When nuclear extracts were incubated with [125 I]T3 and DNA-cellulose, radioactivity remained associated with the DNA-cellulose pellet after extensive washing, consistent with the known interaction of T3 receptors with total DNA (16). When the incubation was performed with heat-inactivated receptors (nuclear extract preincubated for 1 hr at 37°C) or bovine serum albumin (1 mg/ml), specific binding of T3 to DNA-cellulose was abolished, confirming the earlier demonstration (16) that free T3 does not bind to DNA. Receptor-mediated T3 binding to DNA-cellulose was reduced in a dose-dependent way when the incubation was conducted in the presence of soluble competing DNA such as salmon sperm DNA. Plots of the ratio of the radioactivity bound in the absence of competing DNA to that bound in the presence of competing DNA, as a function of the amount of competing DNA added, yielded a straight line, the slope of which is proportional to the affinity of the receptor for the competing DNA fragment (17). For blunt-ended linear fragments of pBR322 DNA between 300 and 4000 base pairs (bp) long, the slope was ≤ 1 (slope units = $10^3 \times B_0/B$ per ng of DNA) (Fig. 2). We then examined the competing ability of a number of nonoverlapping restriction fragments encompassing from –2.8 kilobases (kb) (+1 is the cap site) to +3.9 kb (i.e., well beyond the last exon) of the human GH gene. Similar experiments were conducted with fragments from –2.3 kb to +2.5 kb of the human CS gene. For each gene, the fragment corresponding to the promoter region bound better than the others, with a relative affinity such that the slope was severalfold greater than that for pBR322 DNA (Fig. 2). Depending on fragment length, changes over one order of magnitude in slope units may in fact correspond to changes in affinity over several orders of magnitude (17). Two other regions that bind the receptor with high affinity were found in the CS gene, one between positions –1600 and –1100 and one downstream from the cap site extending to within most of the second exon. The results for these and other fragments studied are shown in Fig. 3.

To better define the binding region in the GH gene, the *EcoRI* (–496)–*BamHI* (+2) fragment was subcloned in pBR322 and digested with restriction enzymes to yield two pBR322-GH hybrid fragments, 723 and 1000 bp long carrying (from 5' to 3' in the gene) 206 (*EcoRI*–*NcoI*) and 367 (*EcoRI*–*AluI*) bp, respectively, of the GH gene promoter (Fig. 4). In this way, fragment length was maintained above 0.3 kb, a size under which any DNA can give artifactually high affinity in the assay used (not shown). Preferential binding was retained with only the second of these overlapping subfragments, thereby localizing receptor binding to within a 161-bp region between coordinates –290 and –129 (Fig. 4). Based on the slope obtained for the 751-bp *SacI*–*BamHI* promoter fragment, we calculated an affinity constant K_d of 2 nM for the receptor-DNA interaction. This value is within the order of magnitude described for the interaction of the cyclic AMP receptor protein with the *lac* promoter in *Escherichia coli* DNA (18). By using a similar two-step strategy with the CS gene, the highest-affinity region was delineated within 300 bp upstream from the cap site (Fig. 4).

The region of the two genes that exhibits the highest affinity for the thyroid hormone receptor (i.e., just upstream from the cap site) also contains binding sites for the human glucocorticoid receptor. In the GH gene, these sites map between positions –290 and +2 (ref. 12). However, in contrast to the thyroid hormone receptor, the glucocorticoid receptor binds better to another region of this gene, within

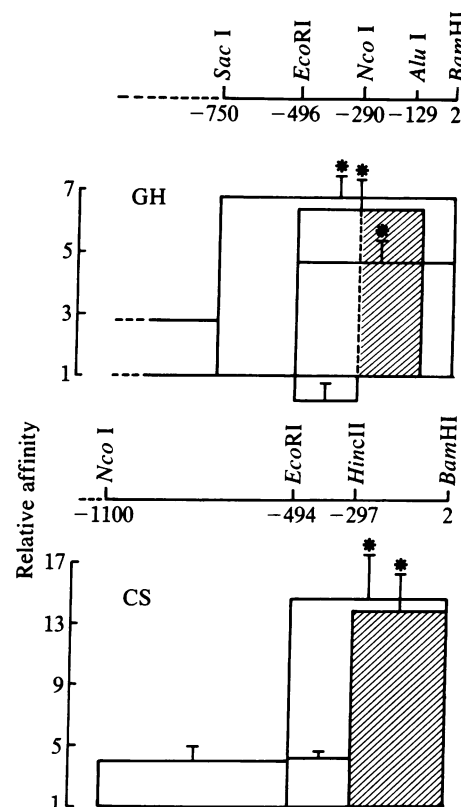


FIG. 4. Binding of the thyroid hormone receptor to the promoter of the GH and CS genes. The DNA restriction fragments indicated were assayed for receptor binding as described in the legends of Figs. 2 and 3. The results shown are means $\pm$ SEM for at least three experiments. An asterisk indicates that this fragment has an affinity significantly ($P < 0.02$) different from that of fragments not designated by an asterisk. The shaded area corresponds to the smallest region containing the preferential thyroid hormone receptor-binding activity. For the GH gene, this area is delineated by the difference between overlapping fragments.

251 bp downstream from the cap site, than to the promoter region (12). This downstream region contains, in the first intron of the gene, a 15-bp consensus sequence that is found in a number of glucocorticoid-responsive genes (10–12). In addition, the human glucocorticoid receptor is known to bind with high affinity to a fragment (LTR451) of the long terminal repeat of mouse mammary tumor virus that contains a glucocorticoid response element (12). We found that binding of the thyroid hormone receptor to LTR451 was no better than to pBR322 DNA (Fig. 3). Finally, it should be noted that the thyroid hormone receptor-containing nuclear extracts used here lack glucocorticoid receptor activity (not shown). Thus, the thyroid hormone receptor-binding region is distinct from the high-affinity glucocorticoid receptor-binding region. Still, the coexistence in the GH gene promoter of binding site(s) for the thyroid hormone receptor and of low-affinity binding site(s) for the glucocorticoid receptor is in accordance with the hypothesis (19) that the transcriptional synergism between thyroid and glucocorticoid hormones in controlling GH gene expression depends on the physical interaction of these two types of receptors at their regulatory sites.

Influence of Thyroid Hormone on Thyroid Hormone Receptor Binding to DNA. Since unoccupied thyroid hormone receptors are associated with chromatin, they could operate as repressors of GH gene transcription (20). The function of thyroid hormone would then be to decrease receptor affinity for DNA, thereby derepressing the gene and allowing its activation (negative control). Our demonstration that T3-bound receptor interacts preferentially with the GH and CS genes promoter does not support this hypothesis. Moreover, we tested whether hormone-free receptor could bind to DNA by running the assay in the absence of T3 and adding [¹²⁵I]T3 afterward to detect receptor bound to DNA-cellulose. Under these conditions, free receptor still interacted with DNA-cellulose, albeit with lower affinity, but was unable to distinguish GH promoter DNA from pBR322 DNA (Fig. 5). Control experiments (results not shown) demonstrated that T3 binding to the receptor is as efficient when the receptor is bound to DNA-cellulose first as when it is free. A diminution

in the ability to bind to the GH promoter preferentially was also observed when the receptor was bound to thyroxine instead of T3, consistent with the notion that thyroxine is a less active substance whose hormonal properties *in vivo* depend predominantly on its conversion to T3 (refs. 2 and 21). These results therefore suggest that T3 renders the receptor capable of recognizing specific gene regions and regulating expression of these genes either directly or by release of a repressor mechanism. This is compatible with the demonstration, in rat pituitary GC cells, that stimulation of GH gene transcription by T3 alone or in synergism with glucocorticoids is proportional to the nuclear concentration of thyroid hormone–receptor complexes (8).

The physiological significance of the interaction between the thyroid hormone receptor and the GH gene promoter reported here is supported by transfection studies (22) that have shown that the 5'-flanking (1.8-kb) region of the rat GH gene confers thyroid hormone responsiveness on the structural gene for xanthine-guanine phosphoribosyltransferase. This "thyroid hormone-response element" has been mapped, by a similar approach, to a region located between positions –235 and +11 in the rat GH gene (23). Thus, this region of the rat GH gene contains the presumptive thyroid hormone receptor-binding site(s). By analogy with the glucocorticoid paradigm, a DNA consensus sequence that recognizes the thyroid hormone receptor might therefore be expected to occur in the rat and human GH genes. This sequence should map between positions –235 (5' boundary in the rat gene) and –129 (3' boundary in the human gene). A comparison of the two genes (13) shows that the best homology is at the 3' end of this area. The nucleotide sequence

*
TG TGGGAGGAGCTTCTAAATTATCCA

from positions –139 to –114 of the human GH gene is indeed perfectly conserved not only in the rat GH gene but also in the human CS gene (to our knowledge, no data are available on the physiological control of the CS gene by thyroid

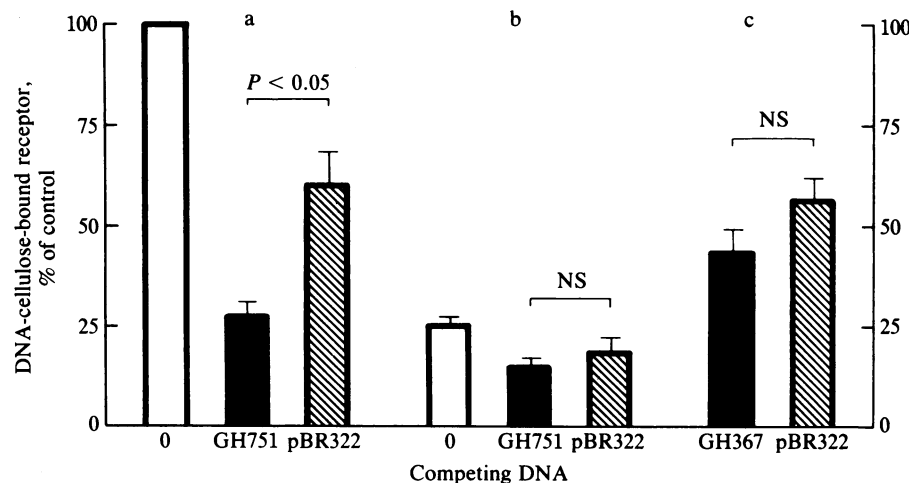


FIG. 5. Influence of the hormone on binding of thyroid hormone receptors to DNA. Incubations were performed as described in the legend of Fig. 2 with the following modifications. Tubes contained nuclear extract, DNA-cellulose, and competing DNA fragment with (series a) or without (series b) [¹²⁵I]T3 or with 0.3 nM [¹²⁵I]thyroxine (series c). After 2 hr at room temperature the three sets of tubes were washed three times with TEDG buffer and the tubes in series a and c were taken for estimation of radioactivity bound to DNA-cellulose. Receptors bound to DNA-cellulose in the remaining tubes (series b) were detected by adding 50 μ l of nuclear extract previously treated at 37°C for 1 hr (a procedure shown to destroy 90% of the receptors), [¹²⁵I]T3 at the same concentration as in the tubes of series a, and buffer to 0.3 ml. After a further 2-hr incubation the DNA-cellulose was washed again and the bound radioactivity was measured. Control experiments showed that total receptor concentration in incubations performed in the absence of T3 (series b) was at least 80% of that in incubations with T3 present (series a). The competing DNA fragments used were GH751 (–750 to +2), 433 ng per tube, and a 4200-bp hybrid of pBR322 and thymidine kinase gene fragment, 404 ng per tube. The competing DNA fragment GH367 was the 1000-bp pBR322-GH hybrid containing the region –496 to –129 of the GH gene promoter, 399 ng per tube. Results (mean $\pm$ SEM for three experiments) are expressed as a percentage of those obtained in the absence of competing DNA (4200–6000 dpm for [¹²⁵I]T3, 2900–3100 dpm for [¹²⁵I]thyroxine). NS, not significantly different.

hormones). This sequence is about 70 bp downstream from the glucocorticoid consensus sequence that could account for the weak binding of the glucocorticoid receptor in the human GH gene promoter (12). It is noteworthy that the 26-nucleotide sequence shown above contains (italicized) one copy, but for one mismatch (asterisk), of the consensus sequence $\text{G TGGGCGG}^{\text{GGC}}_{\text{AAT}}$ of the so-called GC box (24). This "upstream promoter element" is a known target for promoter-specific transcription factors such as the Sp1 eukaryotic protein (24). Also intriguing is the fact that the CS gene promoter, which we find binds the T3 receptor better than does the GH gene promoter, contains an additional such GC-like box, also with a mismatch of the fifth nucleotide, between positions -239 and -230. Whether proteins or factors, other than the thyroid hormone receptor, present in the nuclear extracts play a role in the preferential DNA binding reported here remains to be established.

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