Regulation of Gene Expression by Thyroid Hormone

Herbert H. Samuels,*‡ Barry M. Forman,‡ Zebulun D. Horowitz,* and Zheng-Sheng Ye‡
**Division of Molecular Endocrinology, Department of Medicine, and ‡Department of Pharmacology, New York University Medical Center, New York 10016*

The thyroid hormones (3,5,3',5'-tetraiodo-L-thyronine [L-T4][1] and 3,5,3'triiodo-L-thyronine [L-T3]) have marked effects on the growth, development, and metabolic function of virtually all organ systems and tissues of human beings and other higher organisms (1–3). L-T4 was one of the first hormones to be isolated and its structure determined (4–6). Several decades later L-T3 was identified in the circulation (7–9). Although L-T3 is secreted by the thyroid gland, a significant amount is derived from peripheral conversion of L-T4 to L-T3 by 5'-deiodinases in various tissues (10). L-T3 is about 10-fold more active than L-T4 on a molar basis (3). Because of its higher intrinsic biologic activity, its conversion from L-T4 in various tissues, and its lower affinity for serum binding proteins, L-T3 plays a more dominant role than L-T4 in maintaining the normal euthyroid state (2). Because of the marked alterations in the rate of oxygen consumption identified in hypothyroidism and hyperthyroidism, initial studies on the mechanism of action of the thyroid hormones were focused on mitochondrial function. In vitro studies indicated that iodothyronines could alter the function of isolated mitochondria (1, 11). However, this occurred only at iodothyronine concentrations 100,000-fold higher than those found in the euthyroid state, and L-T3 was less active than L-T4 (11).

In the 1960s Tata and co-workers showed that thyroid hormone administration increased the rate of RNA synthesis in rat liver suggesting that L-T3 and L-T4 might act by controlling gene expression (12, 13). Research over the past decade using intact animals and cultured cells has provided compelling evidence that the thyroid hormones exert their effects in various cells and tissues by stimulating the accumulation of mRNAs which code for specific proteins. The following thyroid hormone-dependent effects have been studied in detail: stimulation of growth hormone synthesis in the rat anterior pituitary in vivo (14); regulation of growth hormone gene expression in cultured rat pituitary cell lines (15–24); stimulation of malic enzyme mRNA in the liver (25–29), as well as several other genes that encode hepatic proteins of unknown function (S_{11} and S_{14}) (30–33); and stimulation of the α-myosin heavy chain gene in the myocardium (34–38). In addition to stimulating the expression of genes, the thyroid hormones also inhibit the expression of certain genes, most notably thyrotrophin (39–41) and the β-myosin heavy chain gene (34–38). Of the genes indicated (both positive and negative regulation), the effect of the thyroid hormones has been shown to occur fully or to a significant degree at the transcriptional level.

Abundant evidence indicates that most if not all of the significant cellular responses regulated by the thyroid hormones in mammalian cells is mediated by a cellular receptor localized to the cell nucleus (2, 3). Evidence to support this notion has been derived from studies in intact animals (2, 33) and cultured cells (3, 23, 42, 43). Several different related strains of growth hormone producing rat pituitary cell lines (GH_1, GH_3, GH_4, and GC) have been shown to be highly effective cell culture models for studying thyroid hormone action. In these cells physiological concentrations of L-T3 and L-T4 stimulate growth hormone synthesis and growth hormone mRNA accumulation (15–24), and the kinetics of stimulation is similar to that found in the anterior pituitary after thyroid hormone injection (14). These cell lines contain thyroid hormone nuclear receptors which have affinity and hydrodynamic properties similar to receptors in various tissues in vivo. A review of the effect of the thyroid hormones on hepatic gene expression by thyroid hormone has recently been published by Oppenheimer et al. (33). In this article we will provide an overview of recent developments in the field of thyroid hormone action that involve cloning of thyroid hormone receptor-related mRNAs and the use of the rat growth hormone gene as a model to identify *cis*-acting DNA sequences and *trans*-acting regulatory proteins required for transcriptional stimulation of the gene by thyroid hormone.

General properties of thyroid hormone receptors

The properties of thyroid hormone nuclear receptors derived from studies using GH_1 and GC cells are listed in Table I. Similar results have been observed with receptor obtained from rat liver nuclei. The affinity of receptors for L-T3, L-T4, and other iodothyronines parallels the iodothyronine biologic potency in cultured cells (42) and in intact animals (44). If the affinity for receptor and the biologic potency of L-T3 is assigned the value of 1, the values for several well-studied iodothyronines are: triiodothyroacetic acid (TRIAC) (3.0) > L-T3 (1.0) > L-T4 (0.10) > 3,3'5'-triiodo-L-thyronine (reverse L-T3) (> 0.01). These relative affinities have been found for receptor in rat brain, liver, and kidney, and for receptor in a variety of cultured cell lines derived from the rat and other species (3, 42, 44, 45). Scatchard analysis of hormone binding to receptor

Address reprint requests to Dr. Samuels, Division of Molecular Endocrinology, Department of Medicine, New York University Medical Center, 550 First Avenue, New York, NY 10016.

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1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; L-T3, 3,5,3'-triiodo-L-thyronine; L-T3-PAL, *N*-2-diazo-3,3,3'-trifluoropropionyl-3,5,3'-triiodo-L-thyronine; L-T4, 3,5,3',5'-tetraiodo-L-thyronine; XGPT, xanthine-guanine phosphoribosyltransferase.

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Table I. Properties of Thyroid Hormone Receptors in GH_1 and GC Cells

Single class of high-affinity binding sites with no evidence for cooperativity.
Iodothyronine affinity parallels biologic potency.
15,000 receptors per cell nucleus.
No cytoplasmic counterpart.
DNA-binding protein.
Receptor is extracted from nuclei by 0.4 M KCl.
Sedimentation coefficient, 3.8 S.
Stokes radius, 3.3 nm.
Estimated molecular weight, 54,000.
Receptor has a half-life of 4.5 h and a synthetic rate of ~ 2,000 molecules/h per cell.

The properties of thyroid hormone nuclear receptors in GH_1 and GC cells indicated in the table are compiled from references (23, 42, 46, 50, 54, 57).

demonstrates linear plots with no evidence for positive or negative cooperativity (46–49). GH_1 and GC cell nuclei contain ~ 15,000–20,000 receptor molecules per cell nucleus (23, 46, 47, 49, 50). The anterior pituitary and rat liver have ~ 8,000–10,000 receptors per cell nucleus while other tissues have lower levels of receptor abundance (51–53). Unlike steroid hormone receptors, no cytoplasmic counterpart of the nuclear receptor has been identified when cells are lysed (47, 54), and these receptors can associate with nuclear components in the absence of ligand (47, 48, 55). Thyroid hormone receptor in nuclear extracts can bind to DNA in vitro (56, 57), and the receptor appears to be predominantly associated with linker DNA regions in chromatin (57–59). The receptor, extracted from nuclei with buffer containing 0.4 M KCl, has a sedimentation coefficient of 3.8 S, a Stokes radius of 3.3 nm, and a particle density of 1.36 g/cm^3, and micrococcal nuclease digestion studies of chromatin indicate that the receptor appears to protect a DNA fragment of about 35 base pairs (57). Using the sedimentation coefficient (3.8 S), the Stokes radius (3.3 nm), and the particle density (1.36 g/cm^3), the molecular weight (M_r) of the salt extracted receptor was estimated to be 54,000 (57). Similar results have also been reported for receptor extracted from rat liver nuclei by high salt (M_r = 50,000, Stokes radius = 3.5 nm) (60). The DNA-binding properties of the receptor is retained when the salt concentration is lowered to 0.1 M KCl (56, 57), and the affinity of salt extracted receptor for iodothyronines (47) is identical to receptor in isolated nuclear preparations (61), suggesting that the interaction of receptor with DNA does not alter its affinity for ligand.

Photoaffinity labeling identifies several molecular weight forms of receptor in GH_1 cells

The structure of the receptor was examined in more detail using a photoaffinity label derivative of L-[^{125}I]T3 (62) in which the amino group of the alanine side chain was derivatized to form a peptide bond with 2-diazo-3,3,3-trifluoropropionate to form *N*-2-diazo-3,3,3-trifluoropropionyl-L-T3 (L-T3-PAL). This compound has ~ $^1/_{10}$ of the affinity of L-T3 for receptor and can be covalently coupled to receptor in intact cells and in solubilized receptor preparations. On exposure to ultraviolet light (254 nm) the 2-diazo-3,3,3-trifluoropropionyl moiety generates a highly reactive carbene intermediate which covalently modifies residues which are sterically favored in the hormone binding domain. Fig. 1 illustrates the SDS-gel electrophoretic profile of receptor photoaffinity labeled using intact GH_1 cells. Lane *1* represents cells incubated with 2 nM L-[^{125}I]T3-PAL while the nuclear extract derived from the cells represented in lane *2* also received a 1,000-fold molar excess of nonradioactive L-T3. L-[^{125}I-T3-PAL covalently modifies an abundant 47,000-M_r component as well as a less abundant 57,000-M_r doublet species and labeling of both components was inhibited by nonradioactive L-T3.

Several lines of evidence indicate that each covalently modified species has properties characteristic of receptor. First, a variety of iodothyronine analogs differentially inhibit the extent of coupling to the 57,000- and 47,000-M_r receptor forms (62) as predicted by their known relative affinities for receptor (42). Secondly, the different molecular weight receptor components generated the same ^{125}I-labeled fragments when peptide mapping was performed with *Staphylococus aureus* V8 protease (24,000 and 12,000 M_r) or trypsin (18,000 M_r) (62). By combining the techniques of photoaffinity labeling (62) and dense amino acid labeling (50), the half-lives and the relative synthetic rates of the 57,000 and 47,000 M_r receptor forms in GH_1 cells were determined (54). These studies indicate that the two M_r receptor forms have similar receptor synthetic rates but have different half-lives (the half-life of the 57,000-M_r form is ~ 2 h while the half-life of the 47,000-M_r form is about 6 h). The following was proposed to explain the

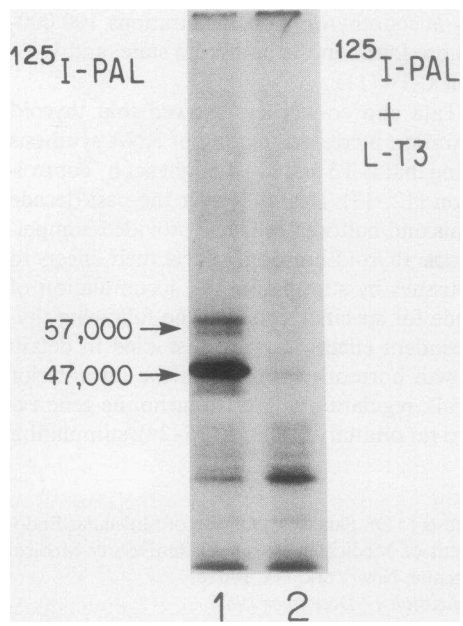

Figure 1. Receptor forms identified by photoaffinity labeling intact GH_1 cells using L-[^{125}I]T3-PAL. Cells were incubated for 1.5 h with 2 nM L-[^{125}I]T3-PAL (*^{125}I-PAL*) alone (lane *1*) or with 5 μM L-T3 (lane *2*). Cell monolayers were then irradiated for 2 min at 254 nm. Nuclear proteins were extracted from nuclei with 0.4 M KCl buffer and the samples were electrophoresed in a 6–15% exponential polyacrylamide-SDS gel. The dried gel was autoradiographed for 36 h at −80°C. Nuclear proteins specifically labeled by L-[^{125}I]T3-PAL had estimated molecular weights of 47,000 and 57,000 (a doublet species). Detailed methods are given in reference 54.

existence of the two thyroid hormone receptor species in GH_1 cells: (*a*) the 57,000-M_r receptor is converted to the 47,000-M_r species in a precursor–product-type relationship and the 47,000-M_r form is more abundant as a result of its longer half-life; (*b*) the 57,000-M_r forms reflect postsynthetic modification of the 47,000-M_r receptor (e.g. phosphorylation, etc.) which could result in anomalous migration in SDS-gels and yield an apparent higher molecular weight; (*c*) the two M_r receptor forms are products of two distinct receptor genes; and (*d*) they result from alternative splicing of the same gene or are generated from the same mRNA by initiation at more than one AUG codon which are in the same open reading frame. Recent studies in which several thyroid hormone receptor-related cDNAs have been cloned supports the view that cells may express more than one thyroid hormone receptor protein.

The thyroid hormone receptor is related to the avian erythroblastosis virus v-erb-A gene

The avian erythroblastosis virus, a defective leukemia retrovirus, induces sarcomas and erythroblastosis in vivo and induces transformation of fibroblasts and erythroblasts to neoplastic phenotypes in vitro (63). Two domains of the avian erythroblastosis virus genome were functionally identified: the v-*erb*-A region and the v-*erb*-B region (63). The v-*erb*-B gene has been shown to encode a truncated constitutively active epidermal growth factor receptor (64–68). Although it had been shown by deletion studies that v-*erb*-B is responsible for the transforming potential of the avian erythroblastosis virus this function required the expression of the v-*erb*-A gene for full oncogenic potential (69–71). A human homologue of the v-*erb*-A gene was identified on human chromosome 17 near the break point found typically in human promyelocytic leukemia, while c-*erb*-B was localized to chromosome 7 (72–74). Using v-*erb*-A sequences as a probe, Vennstrom and Bishop (75) first identified that chick cells expressed a cellular homologue (c-*erb*-A) of the v-*erb*-A gene.

After the cloning of the human glucocorticoid receptor cDNA (76), Weinberger et al. (77) pointed out that a cysteine-rich region of the glucocorticoid receptor showed a high degree of amino acid homology (~ 50%) with a cysteine-rich region of the avian erythroblastosis virus v-*erb*-A gene. With the cloning of other steroid hormone gene receptor cDNAs (e.g., progesterone and estrogen receptor) (78–81), the cysteine-rich region of each receptor was also found to show high homology with the cysteine-rich region of the v-*erb*-A gene. Based on a comparison of the regions of amino acid homology of the human and chick estrogen receptor, Krust et al. (81) divided the domains of the proteins into regions defined as "A," "B," "C," "D," "E," and "F." Between the chick and human estrogen receptors the cysteine-rich C regions showed 100% homology and the long E region at the carboxy-terminal end of the protein showed 94% homology. Other regions showed much lower levels of homology. The C region of the various steroid receptors has been mapped to the DNA-binding region of the receptor (82, 83), and the sequence of the cysteine-rich region is similar but not identical to other regulatory proteins (e.g., TFIIIA) which contain zinc-associated "DNA-binding fingers." However, other regions of the molecule may also be important in influencing the transcriptional response which might mediate protein–protein contact of receptor with other regulatory proteins. The conserved E region of the various steroid receptors appears to contain the ligand-binding domain and shows very low homology between the different receptors (e.g., estrogen and glucocorticoid receptor) (81).

Using v-*erb*-A probes corresponding to the E region of the viral gene, cellular homologues of v-*erb*-A have recently been isolated from a chick embryo cDNA library (75, 84) and a human placenta cDNA library (85). The reticulocyte lysate in vitro translation products of these cDNAs were found to bind L-T3, L-T4, and other iodothyronine analogues with the same relative affinity (84, 85) as found for thyroid hormone nuclear receptor in GH_1 cells and other rat tissues (42, 45). The chick c-*erb*-A encodes a 408-amino acid protein while the human c-*erb*-A isolated from the placental library encodes a 456-amino acid protein. The gene that encodes the human erb-A–related mRNA was localized to chromosome 3 (85) whereas previous studies localized a v-*erb*-A–related sequence to chromosome 17 (72, 73), suggesting that the human genome has at least two *erb*-A–related genes.

SDS-gel electrophoresis of the in vitro translation products of the cloned placental-derived human c-*erb*-A yields 55,000- and 52,000-M_r proteins which likely correspond to the 52,200- and 49,100-M_r proteins that would be initiated by different AUG codons in the same open reading frame as predicted by the nucleotide sequence (85). Translation of the chick c-*erb*-A forms a protein which migrates with an M_r of 46,000 in SDS gels (the nucleotide sequence predicts a protein with an M_r of 46,700) (84). Unlike the cloned chick c-*erb*-A, the v-*erb*-A gene product (75,000 M_r containing viral *gag* sequences at the amino terminus) does not to bind thyroid hormone (84). However, it is a DNA-binding protein, suggesting that it might interact with functional sequences to block or constitutively activate gene expression (84). Both the human and chick c-*erb*-A clones show high homology within the putative DNA binding C region (68 amino acids) and by analogy to steroid hormone receptor cDNAs, the putative ligand-binding E region (206 amino acids) (84, 85). However, other regions of the c-*erb*-A cDNAs are less conserved and it has been suggested that the cloned chick c-*erb*-A is more related to the *erb*-A gene on human chromosome 17 rather than chromosome 3 (85).

Photoaffinity labeling was used to identify the receptor forms in human cells and the results are shown in Fig. 2 along with a parallel experiment using GH_1 cells (86). Several M_r forms of thyroid hormone receptor in human fibroblasts (lanes *1* and *2*) were identified which are not identical but are in the same molecular weight range as the receptor in GH_1 cells (lanes *3* and *4*). The receptor species in fibroblasts are much less abundant than receptor in GH_1 cells and the photoaffinity-labeled human receptor species were estimated to have M_r values of 55,000, 52,000, and 45,000 with the 52,000-M_r form being the most abundant. Two of these species may correspond to the M_r forms generated by in vitro translation of the cloned human c-*erb*-A mRNA (55,000 and 52,000 M_r) which appear to reflect initiation at two distinct AUG codons in the same open reading frame (85). The 45,000-M_r photoaffinity-labeled receptor in human cells may reflect expression of a different *erb*-A gene, such as the one on chromosome 17, which may be related to the chick c-*erb*-A which encodes a 46,200-M_r protein.

At the time that this article was written, two other c-*erb*-A–related cDNAs have been cloned and sequenced (87, 88). Thompson et al. (87) reported the cloning of a c-*erb*-A–related cDNA from a whole rat-brain cDNA library which codes for a protein of 410 amino acids. The age, strain, and sex of the rat,

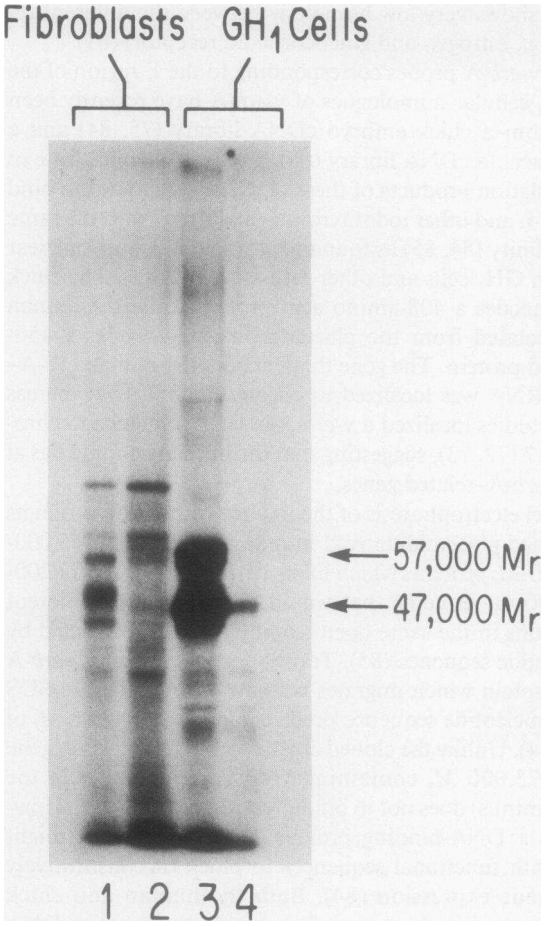

Figure 2. Receptor forms identified by photoaffinity labeling intact human fibroblasts (lanes *1* and *2*) and GH_1 cells (lanes *3* and *4*). Cells were incubated for 1.5 h with 2 nM L-[^{125}I]T4-PAL alone (lanes *1* and *3*) or with 5 μM L-T3 (lane *2* and *4*). Cell monolayers were then irradiated for 2 min at 254 nm. The extracted nuclear proteins were electrophoresed, and the dried gel was autoradiographed at −80°C for 48 h. This figure was taken from Horowitz et al. (86).

however, were not indicated. In contrast with the *erb*-A–related cDNA isolated from the human placental cDNA library, the rat brain *erb*-A–related cDNA showed preferential hybridization to human chromosome 17. The rat brain *erb*-A cDNA showed greater homology with the chick c-*erb*-A in the putative DNA binding C region (97%) and the putative ligand-binding E region (94%) than in the comparable regions of the human placental c-*erb*-A (90% in the C region and 85% in the E region). Hybridization studies with the rat brain c-*erb*-A using total RNA from a variety of tissues and cells identified an abundant 2.6-kb mRNA and less abundant mRNA species of about 5.0 and 6.0 kb in brain, kidney, gut, heart, and to a lesser degree in lung, testes, and spleen but not in liver, suggesting that this *erb*-A–related mRNA is expressed in a tissue-specific manner. However, hybridization to poly(A^+) mRNA from liver was not reported, and the liver RNA was not hybridized to other *erb*-A probes documenting that *erb*-A–related mRNAs could be identified in the liver RNA preparation. In addition to the apparent low expression of an mRNA related to the brain *erb*-A cDNA in liver, the relative affinity of the translated *erb*-A protein for TRIAC, L-T3, and L-T4 was reported to be different from that of the endogenous receptor studied in liver and in GH_1 cells in that the affinities for TRIAC and L-T3 were identical and the affinity for L-T4 was ~ 50-fold lower than the other two compounds (87). These relative affinities, however, differ from that reported to occur in rat brain nuclei (45), suggesting that the cloned rat-brain cDNA protein is not significantly expressed in brain or is expressed in a small subset of brain cells so that it would not be detected when analyzing whole-brain nuclei.

Benbrook and Pfahl (88) recently isolated a c-*erb*-A clone from an adult human testes library which encodes a protein of 490 amino acids and shows greater homology with the chick c-*erb*-A in the putative DNA-binding C region (96%) and the putative ligand-binding E region (94%) than in the comparable regions of the human placental c-*erb*-A (87% in the C region and 84% in the E region). The high homology with the chick c-*erb*-A protein suggests that this human c-*erb*-A may be a product of the previously identified v-*erb*-A–related gene on human chromosome 17. However, the affinity of only L-T3 was reported so it is not known whether the ligand-binding properties of this human c-*erb*-A is similar to or different from that reported for the other cloned *erb*-A–related cDNAs isolated from chick embryo, human placenta, and rat brain.

The identification of several *erb*-A thyroid hormone receptor–related cDNA clones have been interpreted to suggest tissue-specific or functional heterogeneity of thyroid hormone receptors (87). In our view, however, the information available is too premature to draw these conclusions. First, as of yet no information is available indicating that these cloned *erb*-A proteins regulate gene expression in a functionally different fashion or influence the expression of different genes. Secondly, two of the cDNAs have been isolated from embryonic tissues and may not be expressed in the neonatal or adult organism. Lastly, all of the cloned *erb*-A cDNA contain multiple small open reading frames prior to the correct translation initiation site which markedly decreases the efficiency of translation of the *erb*-A proteins in vitro (84, 85, 87, 88). The role of these small open reading frames is unclear but raises the possibility that posttranscriptional mechanisms may determine the efficiency of translation of a specific *erb*-A–related receptor mRNA. Therefore, the fact that a specific size *erb*-A mRNA is more abundant than other *erb*-A–related species does not necessarily indicate that it codes for the nuclear associated receptor detected in a particular cell or tissue. This will require the cDNA cloning of the different size *erb*-A–related mRNAs in various tissues followed by the development of specific antibody probes to assess the level of expression the protein encoded by each of the *erb*-A–related mRNAs. Nevertheless, cloning of these *erb*-A mRNAs represents a major advance in the field of thyroid hormone action and should facilitate our understanding of how the receptor functions to transcriptionally regulate gene expression.

DNA sequences of the rat growth hormone gene that mediate basal and regulated expression by thyroid hormone

Studies of a number of thyroid hormone-responsive genes indicate that regulation occurs fully or in part at the transcriptional level, and this is true for genes in which L-T3 stimulates (e.g., rat growth hormone) (21–23) or inhibits (e.g., thyrotrophin) (39–41) expression. The transcription rate of the growth hormone and thyrotrophin genes are modulated within minutes of thyroid hormone exposure (23, 40). Furthermore, changes in the rate of transcription of these genes parallel the

level of nuclear associated hormone–receptor complexes (23). These results support the notion that the thyroid hormone–receptor complex binds to *cis*-acting DNA sequences (referred to as a thyroid hormone response element) which influences the transcription rate of the gene. Virtually all thyroid hormone-responsive genes (e.g., rat growth hormone, thyrotrophin, S_{14}, malic enzyme, and the α-myosin and β-myosin heavy chain genes) are regulated in a cell- or tissue-specific fashion. Therefore, in addition to defining the location of a thyroid hormone response element(s) it is also essential to identify the *trans*-acting factors and *cis*-acting elements involved in mediating cell-specific expression to fully understand how thyroid hormone modulates the expression of these genes.

Since the sequence of the rat growth hormone gene is known and cultured rat growth hormone-producing cells are available for transfection, current information regarding the *cis*-acting elements and *trans*-acting factors involved in thyroid hormone-regulated expression have come from studies of the rat growth hormone gene (89–99). However, as discussed below, some disagreement exists concerning the location of the *cis*-acting sequences of the rat growth hormone gene that mediate cell-specific and thyroid hormone-regulated expression. The location and sequence of the regulatory elements identified in the various studies (89–99) is illustrated in Fig. 3. Functional identification of these DNA elements have been derived from stable and transient transfection studies using chimeric plasmids in which 5′-flanking gene sequences have been linked to reporter genes. The xanthine-guanine phosphoribosyl transferase gene (XGPT) (89) and the neomycin resistance gene (neo) (93, 94) have been used as reporter genes for stable transfection studies, while the chloramphenicol acetyl transferase gene (CAT) has been used for analysis by transient transfection (90–92, 95–99). The chimeric plasmids containing varying lengths of rat growth hormone 5′-flanking DNA linked to these reporter genes have been respectively referred to as pGH-xgpt, pGH-neo, and pGH-cat.

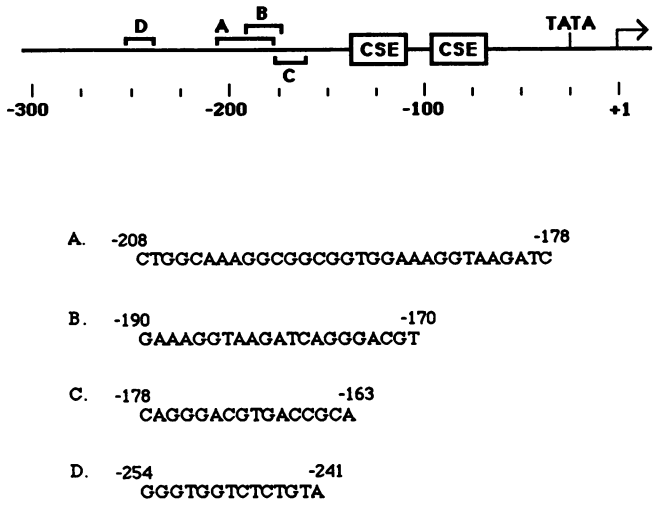

Figure 3. Cis-acting elements in the 5′-flanking region of the rat growth hormone gene. The sequences thought to be involved in mediating cell-specific basal expression (−137/−107 and −95/−65) are indicated by the two boxed regions and are designated as *CSE*. Evidence that these two elements mediate cell-specific basal expression come from functional studies and nuclease footprinting which indicate that a cell specific protein(s), found only in cells which express the rat growth hormone gene, binds to these regions (95, 96, 99, 101–104). Not shown in the figure is a silencer element reported by Larsen et al. (92) which was mapped to sequences between −554 and −237. DNA in this region is thought to mediate cell-specific expression by supressing the activity of the gene in heterologous cells but not in cells that express the rat growth hormone gene (92). Also shown are sequences (indicated as *A, B, C,* and *D*) which have been reported to contain elements (fully or in part) that functionally behave as thyroid hormone response elements. These sequences come from the following studies: Ye et al. (99) (sequence *A*); Koenig et al. (97) (sequence *B*); Glass et al. (98) (sequence *C*); and Wight et al. (94) (sequence *D*).

Stable transformation studies

Using a pGH-xgpt plasmid containing 1,800 bp of 5′-flanking DNA of the rat growth hormone gene [pGH-xgpt(−1,800)], Casanova et al. (89) showed that L-T3 stimulated XGPT enzyme and XGPT mRNA in GC cells which were stably transformed by this vector. S_1 nuclease mapping indicated that the 5′-flanking region of the rat growth hormone gene directed accurate transcriptional initiation of the gene as well as thyroid hormone-regulated expression (89). L-T3 stimulated similar relative changes (10- to 20-fold) in the expression of both the integrated pGH-xgpt(−1,800) gene and the endogenous growth hormone gene at both the mRNA and the transcriptional level (89, 95). In contrast, when the rat growth hormone gene was used to transfect heterologous cells, correct transcriptional initiation did not occur (100). These results indicated that the first 1,800 bp of 5′-flanking DNA of the rat growth hormone gene contains DNA elements which are sufficient to account for the extent of thyroid hormone-regulated expression of the endogenous gene. Furthermore, accurate transcriptional initiation of the integrated pGH-xgpt gene in GC cells (89), compared with stable transformation studies using heterologous cells (100), suggest that rat growth hormone producing cells contain *trans*-acting factors which dictate cell-specific expression of the gene. Using a series of pGH-neo deletion mutants to stably transform GC cells, Crew and Spindler (93) concluded that the elements that mediated thyroid hormone-regulated expression were contained within the first 235 bp of 5′-flanking DNA. However, more recent stable transformation studies from the same laboratory indicated that the DNA sequence that mediated thyroid hormone stimulation of the gene was located between −254 and −241 (Fig. 3, sequence *D*) (94). This result differs from those obtained using transient gene expression which are described below.

Transient gene expression studies

A potential problem in using stable transformation to map DNA regulatory elements is that expression of the transfected gene may be influenced by genomic sequences near the site of integration. This problem is minimized in transient transfection studies. Transient gene expression is examined within 48–72 h after DNA transfection at which time the plasmids primarily exist as minichromosomal elements and are not integrated into the host cell genome. Larsen et al. (91) initially reported thyroid hormone regulation studies in which GC cells were transiently transfected using calcium-phosphate precipitation with various pGH-cat plasmids in which the 5′-flanking DNA was derived from the hooded rat gene. When GC cells were transfected with a pGH-cat plasmid extending to −1,753 bp, L-T3 was found to stimulate CAT gene expression 2.8-

fold. The extent of CAT stimulation by L-T3 increased to four- to fivefold with deletions to −237 and −211, and then fell to about twofold with a deletion to −202. No stimulation was observed with pGH-cat constructs extending to −183 and −137 suggesting that sequences between −211 and −183 were important in mediating thyroid hormone-regulated expression. Flug et al. (95), using the Sprague-Dawley rat gene, studied a series of pGH-cat vectors containing 5'-flanking deletions extending to −1,800, −530, −312, −236, −208, −181, −145, and −104. Basal CAT expression was similar for all the deletion mutants extending from −1,800 to −145, while the extent of basal expression with the −104 pGH-cat deletion mutant was about threefold lower. L-T3 stimulated CAT gene expression to a similar maximal level (~ 10- to 15-fold) in the deletion mutants extending from −1,800 to −208. The extent of L-T3 stimulation sharply decreased with a deletion to −181 (two- to threefold), and deletion to −145 and −104 eliminated thyroid hormone stimulation. These results are in general agreement with those of Larsen et al. (91) except that some L-T3 stimulation was found with the −181 deletion. These results suggest that a strong thyroid hormone response element is localized between −208 and −181 while a second but weaker functional element is located 3' of −181 bp.

The study by Flug et al. (95) also suggested that sequences within the first 145 bp of 5'-flanking DNA are important in mediating cell-specific "basal" expression. None of the pGH-cat deletion mutants were found to be expressed in heterologous cells which included Rat2 fibroblasts, H4 rat hepatoma cells, AtT-20 mouse pituitary cells which express proopiomelanocortin, or monkey kidney epithelial cells (CV-1 and Cos 7) (95, 99). In contrast, GC cells and GH_4C_1 cells showed basal CAT expression with the −145 and −104 deletion mutants (95, 99). The three- to fourfold higher degree of basal expression of the −145 compared with the −104 deletion mutant indicated that sequences between −145 and −104, which are highly conserved among the rat, human, and bovine growth hormone genes, play an important role in enhancing the extent of basal expression of the gene (95). This conclusion is further supported by studies that indicate that a cell-specific nuclear protein, found only in rat growth hormone-producing cells, generates a DNase I footprint between −95 and −65 (96, 99, 101) and between −137 and −107 (99, 102). The location of these cell-specific DNA elements (indicated as *CSE*) is shown in Fig. 3. Similar results were reported for the human growth hormone gene (103), and this protein was found to enhance transcription of the human gene in an in vitro HeLa cell transcription system (104).

The distance between the two footprinted regions, which center at −80 and −122, is exactly four helical turns of DNA. This indicates that the proteins that interact with these regions reside on the same side of the DNA helix favoring protein–protein interactions. By altering the helical and spatial relationship between the −95/−65 and the −137/−107 regions, Ye et al. (99) have provided evidence that the enhanced level of cell-specific basal expression of the −145 vs. the −104 promoter involves an interaction of the proteins that bind to the two regions. Evidence has been presented that the protein–DNA complexes that form in the two regions may be generated by the same protein, or by proteins which recognize a similar sequence motif (TAAAT) found at the center of the two footprinted regions (99, 103). Therefore, studies with both the rat and human growth hormone genes strongly support the notion that the −137/−107 and −95/−65 regions (Fig. 3) function as elements which mediate cell-specific basal expression of these genes. These and other studies by Nelson et al. (90) using other heterologous cells and a pGH-cat plasmid extending to −235, differ with recent studies by Larsen et al. (92) who reported that a "silencer" element, located between −554 and −237, is responsible for the suppression of rat growth hormone gene expression in heterologous but not homologous cells. The reason for this discrepancy is unclear and requires additional investigation.

Both cell-specific and thyroid hormone response elements are involved in thyroid hormone stimulation of rat growth hormone gene expression

Flug et al. (95) examined whether the highly conserved region between −145 and −104 was essential for thyroid hormone-regulated expression by studying L-T3 regulation of internal deletion mutants in which upstream sequences (e.g., −530/−237 and −236/−146) were ligated to the −104 promoter region. Only the −236/−146 region conferred L-T3 regulated expression to the −104 promoter, indicating that sequences upstream of −236 and between −146 and −104 are not essential for regulated expression by thyroid hormone. In a more recent study, Ye et al. (99) found that DNA from −236/−178 conferred full L-T3 regulation to the −104 rat growth hormone gene promoter and the sequence (−236/−178) functioned in either normal or inverted orientations. No significant L-T3 stimulation resulted when a sequence from −181 to −146 was ligated in either orientation to the promoter, while full L-T3 stimulation occurred when only a 31-nucleotide fragment extending from −208 to −178 (Fig. 3, sequence *A*) was ligated to the −104 gene promoter (99). In contrast with the −104 homologous rat growth hormone promoter, sequences between −236 and −146 did not confer L-T3 regulation to an enhancerless SV40 viral promoter, indicating that the upstream region necessary for thyroid hormone-regulated expression functions most efficiently with its homologous promoter (95). Similar results were also found using an enhancerless Rous sarcoma viral promoter (99). However, regulated expression of these heterologous promoters occurred if sequences containing the cell-specific basal elements were ligated to the foreign promoter along with the upstream thyroid hormone response element, and this occurred independently of the orientation of these elements (99). These results suggested that the thyroid hormone and cell-specific elements of the rat growth hormone gene function as an enhancerlike unit, and are both required to confer efficient thyroid hormone regulated expression to heterologous promoters. Ye et al. (99) proposed that thyroid hormone stimulates gene expression by acting via its receptor to enhance the function of the cell-specific basal element(s). This model proposed that the receptor binds to the −208/178 region (99), and that the L-T3-receptor complex acts to "stabilize" or "enhance" the protein–DNA interactions of the cell-specific basal element to form a more "active" transcription complex which stimulates the level of gene expression.

Protein–DNA interactions of sequences that function as thyroid hormone response elements

To clarify the above or other models of thyroid hormone regulation of gene expression, it is important to document that the receptor binds to the sequences which function as a thyroid

hormone response element. A direct interaction of receptor with these sequences would strengthen the notion that the L-T3 receptor complex directly activates the gene, and that stimulation is not mediated by another gene product stimulated by L-T3. In a recent study, Koenig et al. (97) ligated sequences from −210 to −126 and from −237 to −139 to the rat growth hormone promoter extending to −137. Both recombinants showed stimulation by L-T3. However, no stimulation was found when sequences between −190 and −172 were deleted from these fragments, suggesting that a thyroid hormone response element was fully or in part localized to this region (Fig. 3, sequence *B*). Using a partially purified thyroid hormone receptor preparation from rat liver (0.2%) and a −237/−56 fragment of the hooded rat gene, methylation interference was used to attempt to footprint the receptor binding region (97). Although, methylation of two sites (−185 and −186) decreased protein binding, the major region where methylation interference prevented protein binding was at −176 to −174 and at −172 and −171. These results are in keeping with the −190 to −172 internal deletion mutants reported by Koenig et al. (97), but the major sites of methylation interference (−176 to −174, and −172 and −171) are outside of the sequence (−208/−178) (Fig. 3, sequence *A*) which Ye et al. (99) found to contain a functional thyroid hormone response element. Since the liver receptor preparation used in the methylation interference study was only 0.2% pure, it was not possible to verify that the protein–DNA complex identified was formed by receptor. However, the thyroid hormone response elements identified by the internal deletion mutant studies of Koenig et al. (97) (−190 to −172) and by Ye et al. (99) (−208 to −178) suggests that a functionally important region (fully or in part) resides between −190 and −178.

In contrast with the above studies, Glass et al. (98) recently suggested that the thyroid hormone response element of the rat growth hormone gene is localized between −177 and −166 (Fig. 3, sequence *C*). This is based on the observation that a −235/−45 fragment of the gene could confer L-T3 stimulation to a −107 herpes viral thymidine kinase promoter, while no stimulation was found when sequences from −177 to −166 were internally deleted. When DNA from −186 to −158 was linked to the thymidine kinase promoter, moderate L-T3 stimulation of expression was found (2.7-fold). In contrast with these results, Ye et al. (99) found that DNA from −208 to −178 conferred full L-T3 regulation (about 10-fold) to its homologous −104 promoter while no significant L-T3 stimulation (1.5-fold) occurred with sequences from −181 to −146. In support of their functional results, Glass et al. (98) identified a DNase I footprint extending from −178 to −163 (Fig. 3, sequence *C*), using 8 fmol of a DNA fragment and a salt extract of GC cell nuclei (containing 12 fmol of receptor) which was not further purified. In these extract preparations, which contain an abundance of DNA binding proteins, the receptor represents ~ 0.002% of the total protein. The impurity of the receptor preparation, and the lack of a control such as nuclear extract from cells with very low levels receptor (e.g., rat2 cells), however, raises the possibility that the −178/−163 footprint is formed by a nuclear protein other than the receptor.

Using streptavidin-agarose, oligonucleotide probes (−209/−146 and −186/−158) containing biotin-11-dUTP were also used to measure the binding of L-[^{125}I]T3 receptor from GC cell nuclear extracts and in vitro synthesized ^{35}S-labeled human c-*erb*-A protein (98). Using 100 fmol of biotinylated oligonucleotide ~ 5 fmol of receptor from a 25-μl GC cell extract and 1–2 fmol (1,000 cpm) of ^{35}S-labeled human *erb*-A protein bound to these fragments. 4 μl of translated reticulocyte lysate was used in the binding reaction. Although the amount of in vitro translated *erb*-A protein in the binding reaction was not given, 4 μl of lysate would be expected to incorporate at least 200,000 cpm of [^{35}S]methionine into the protein, indicating that only ~ 0.5% (1,000/200,000) of the input human *erb*-A bound to the oligonucleotides. This implies that the human *erb*-A protein has a very low intrinsic affinity for these sequences. Alternative explanations are that the protein does not form the correct tertiary structure required for DNA binding even though it binds hormone, or that some type of intracellular postsynthetic modification, or the presence of other proteins, enhances the affinity of the human *erb*-A protein for DNA. The relative affinity of GC cell L-[^{125}I]T3 receptor for the −209/−146 fragment was reported to be two to threefold higher than for the −186/158 oligonucleotide. Although this difference in affinity implies that thyroid hormone receptor binding might also occur between −209 and −186, this interpretation was discounted since a −235/−177 oligonucleotide did not bind appreciable amounts of receptor (98). However, this result does not exclude the possibility that the receptor recognition sequence is at the 3′ end of the −235/−177 region (e.g., from −190 to −177), since deletion of DNA 3′ of −177 might reduce the number of overall protein-DNA contact points and lower the affinity of receptor for its cognate sequence.

Future directions

Taken together, the studies of Larsen et al. (91), Flug et al. (95), Koenig et al. (97), Glass et al. (98), and Ye et al. (99) indicate that DNA between −209 and −166 functions as a thyroid hormone response element which is involved in regulated expression of the rat growth hormone gene. The difference in the location of this element(s) identified by these transient expression studies (91, 95, 97–99), and also the element reported by Wight et al. (94) (−254/−241) using stable transformation (Fig. 3), raises the possibility that several regions may be able to function as a thyroid hormone response element, depending on the location of these sequences relative to other regulatory elements of the promoter element, and the nature of the promoter used for functional analysis. More definitive studies are required to fully document the precise location of the sequence that functions as the dominant thyroid hormone response element of the gene. This will require constructing a series of point or multiple mutations across the −254/−166 region of the gene using the 5′-flanking region containing sequences from −254 to +7. Analysis of the function of these recombinants coupled with footprinting studies of these constructions using purified receptor in vitro are required to precisely identify the DNA sequences required for thyroid hormone regulated expression of the rat growth hormone gene.

Precise identification of the region(s) required for thyroid hormone regulated expression of the rat growth hormone, however, will not provide a complete picture of how the thyroid hormone–receptor complex activates or inhibits gene expression. As indicated above, the results of Ye et al. (99) suggested that both an upstream thyroid hormone response element and a cell-specific basal element are necessary for L-T3 regulation of the rat growth hormone gene. To account for these observations a mechanism for L-T3 stimulation of ex-

pression of the rat growth hormone gene was proposed in which receptor and cell specific protein–protein interactions occur. In this model it is assumed that the receptor binds to its cognate sequence and the L-T3 receptor complex acts to stabilize or enhance the protein–DNA interactions of the cell-specific basal element to form a more active transcription complex which increases the level of gene expression. This model can be tested by in vivo footprinting of the cell-specific elements of the endogenous rat growth hormone gene after L-T3 incubation. In vivo footprinting might also determine whether the thyroid hormone receptor binds to the same sequences in situ as determined by footprinting studies using purified receptor in vitro.

Recent studies on the mouse mammary tumor virus (MMTV) promoter support an analogous two-element model for glucocorticoid hormone stimulation of gene expression. Inactivation of the binding site for the NF-I transcription factor (or a protein with a similar recognition sequence; e.g., TGGCA-binding protein) markedly lowers stimulation by glucocorticoid hormones without significantly altering the basal activity of the gene (105, 106). Furthermore, in vivo footprinting indicates that glucocorticoid hormone incubation increases the association of NF-I with its cognate sequence (107) suggesting that the glucocorticoid receptor enhances NF-I binding or interacts with NF-I to form a transcription complex which activates expression of the MMTV promoter. Since the control elements of thyroid hormone-responsive genes other than rat growth hormone have not yet been reported, it is not possible to determine whether two elements are necessary for efficient L-T3–regulated expression in other systems. However, it provides a mechanism to explain how thyroid hormone, presumably acting via the same receptor, can positively (e.g., rat growth hormone) or negatively (e.g., thyrotrophin) regulate gene expression. Whether positive or negative regulation occurs would be dependent on the hormone–receptor complex acting in *cis* to enhance or suppress the effect of a second *trans*-acting regulatory protein(s) that plays a central role in determining the rate of expression of the gene.

Additional studies are required to support this hypothesis since positive and negative regulation may be mediated by structurally similar but different thyroid hormone receptors. The observation that the human genome contains at least two *erb*-A–related genes on different chromosomes raises this possibility (72–74, 85). Significant progress has been made in the area of thyroid hormone action since the initial identification of thyroid hormone nuclear receptors in liver and kidney (52) and in cultured cells (46). Future advances in this field will require the cloning of thyroid hormone receptor mRNAs from various tissues and cells to identify their structure and function. In addition, other thyroid hormone response genes are being isolated and sequenced. Identification of the thyroid hormone response elements of other genes and the regulatory proteins that mediate their expression should ultimately provide a comprehensive view of the detailed mechanisms involved in positive and negative regulation of thyroid hormone-responsive genes in various cells and tissues.

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