An important physiological control of the glycoprotein hormone \( \alpha \)-subunit is the negative feedback by thyroid hormones in the thyrotrope. A region of the rat glycoprotein hormone \( \alpha \)-subunit gene that is involved in transcriptional regulation by thyroid hormone has been identified by transient transfection studies, and sequence-specific binding of the thyroid hormone receptor to a site within this region has been demonstrated. Deletion-mutation studies using plasmid expression vectors containing either 246, 170, or 80 base pairs of the 5'-flanking region of the rat \( \alpha \)-subunit gene fused to the coding region of the bacterial chloramphenicol acetyltransferase gene demonstrate 3,5,3'-triiodo-l-thyronine (\( T_3 \))-regulated expression in GH3 cells, a \( T_3 \)-responsive somatotroph cell line. In order to investigate the possibility of thyroid hormone receptor interaction with this segment of the rat \( \alpha \)-subunit gene, the binding of the thyroid hormone receptor to synthetic oligodeoxynucleotides was analyzed using an avidin-biotin complex DNA binding assay. An oligodeoxynucleotide representing a fragment of the \( \alpha \)-subunit gene from -74 to -38, relative to the transcriptional start site, shows significant binding to \( [125]T_3 \)-receptor complex present in nuclear extracts of GH3 cells. This fragment binds receptor to a degree similar to that seen with a fragment of the rat growth hormone gene which contains a putative thyroid hormone-responsive element. In addition, this fragment of the rat \( \alpha \)-subunit gene binds to the in vitro synthesized human \( c \)-erb\(A_\beta \) protein, which has been identified as a member of the family of putative \( T_3 \) receptors. These data demonstrate that a cis-active thyroid hormone-responsive element resides in the 5'-flanking region of the rat \( \alpha \)-subunit gene and that the mechanism involved in the suppression of expression of this gene by \( T_3 \) could involve specific binding of the thyroid hormone receptor to this region of the gene.

A single gene encodes the common \( \alpha \)-subunit of the pituitary glycoprotein hormones (luteinizing hormone, follicle-stimulating hormone, and TSH\(^1 \)) and placental chorionic gonadotropin. The expression of the \( \alpha \)-subunit gene is regulated by several factors, including thyroid hormone (\( T_3 \)), thyrotropin-releasing hormone, cAMP, sex steroids, and GnRH (1-7). The rat \( \alpha \)-subunit gene is a useful model for the study and identification of multiple hormone regulatory cis-elements, since several of these factors act at the transcriptional level. For example, cAMP stimulates the transcription of the human \( \alpha \)-subunit gene (8, 9), and this effect appears to be mediated through a cis-acting enhancer element located in the 5'-flanking region (10-12). Thyroid hormone acts to regulate negatively the levels of rat \( \alpha \) and TSH\(\beta \)-subunit mRNAs in the pituitary gland, in large part, by suppressing the transcription of both the \( \alpha \) and TSH\(\beta \)-subunit genes (13-18). The effect on transcription is rapid, occurs in vivo and in primary cultures of pituitaries, and does not require ongoing protein synthesis, suggesting that \( T_3 \) directly regulates the activity of the \( \alpha \)-subunit gene. \( T_3 \) is believed to regulate the transcription of other genes (19, 20), the best characterized of which is the stimulation of rGH transcription which is apparently mediated by the binding of \( T_3 \) to a chromatin-associated nuclear receptor (21). Studies from several laboratories have localized the binding region for the \( T_3 \) receptor within the 5'-flanking region of the rGH gene (22-25). However, identification and agreement on the precise rGH gene sequence required for \( T_3 \) binding have not been obtained, and binding to specific sequences has not been reported for any other \( T_3 \)-regulated gene.

To further our understanding of the molecular mechanisms involved in the regulation of \( \alpha \)-subunit gene expression and to identify sequences in this gene required for the inhibition of expression by \( T_3 \), we have examined \( T_3 \)-regulated expression of chimeric plasmids in which portions of the \( \alpha \)-subunit gene promoter directs expression of chloramphenicol acetyltransferase gene (CAT) transfected into GH3 cells, a \( T_3 \)-responsive cell line. These studies have localized a TRE between bases -80 to +33 of the rat \( \alpha \)-subunit gene. To examine possible mechanisms of regulation, the specific binding of nuclear \( T_3 \)-receptors from GH3 cells to this part of the gene was analyzed using the avidin-biotin DNA complex assay (25). In addition, the binding of the human \( c \)-erb\(A_\beta \) gene product, which has been identified as a member of the family of putative thyroid hormone receptors (26, 27), to this DNA has been examined. The apparent binding site of these receptor preparations has been localized to bases -74 to -38.

\(^1\) The abbreviations used are: TSH, thyroid-stimulating hormone; CAT, chloramphenicol acetyltransferase; rGH, rat growth hormone; GnRH, gonadotropin-releasing hormone; HEPEES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; \( T_3 \), 3,5,3'-triiodo-l-thyronine; \( T_3 \)R, thyroid hormone receptor; TRE, thyroid hormone-responsive element; EGTA, ethylenebis(oxyethylenetriitol)tetraacetate acid; bp, base pair(s).
by use of functional studies and measurement of direct binding of the T3R to DNA, we have isolated a small region of the a-subunit gene which promotes thyroid hormone-sensitive transcription and interacts with the receptor.

**MATERIALS AND METHODS**

**Plasmid Constructions—** A promoterless plasmid containing the chloramphenicol acetyltransferase coding region inserted at the HindIII site of the pUC13 plasmid (pUC13CAT) was a gift from Dr. R. H. Goodman (Tufts University, Boston, MA). An expression vector containing the herpes simplex thymidine kinase promoter fused to the CAT gene was generously supplied by Dr. W. Rutter (University of California San Francisco). Plasmids and constructs were prepared using standard techniques (28). The rat a-subunit gene was isolated and sequenced in this laboratory from a rat genomic DNA library (partial HaelIII, a Charon 4A; gift of Dr. Benner, California Institute of Technology, Pasadena, CA) using an a-subunit cDNA probe (29).

**Cell Culture, DNA Transfections, and CAT Assays—** GH3 cells (ATCC CCL 82.1) obtained from Dr. R. Koenig (University of Michigan, Ann Arbor, MI), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml), streptomycin (100 mg/ml), and 2 mM L-glutamine. Cells (2-3 × 10^6) were seeded in 60-mm dishes 24 h prior to transfection with 5-10 pg of DNA in DEAE-dextran (200 pg/ml). Following 2 h of transfection, cells were subjected to MSO shock (30). Cells were then incubated in media containing either thyroid- and steroid-hormone-depleted (31) or hypothyroid calf serum (Rockland Labs, Gilbertsville, PA). In these experiments media were supplemented with 10^{-10}-10^{-8} M T3 and 10^{-8} M dexamethasone; media containing hypothroid serum were supplemented with 10^{-10} or 10^{-8} M T3. The type of serum used in each set of experiments is described in the figure legends. Similar results were obtained with either serum preparation. After 48 h, cell extracts were prepared and assayed for CAT as described (32) using [dichloroacetyl-1,2-14C]chloramphenicol (Du Pont-New England Nuclear). The Hc-erbA protein in reticulocyte lysate mixtures was desalted by gel filtration in binding buffer which also removed unincorporated T3. Incorporation of [35S]methionine into protein was determined by incubation of an aliquot of desalted lysates in 0.1 N NaOH at 37 °C for 10 min to destroy tRNA charged with [35S]methionine, followed by precipitation with 4 volumes of 25% trichloroacetic acid, 2% acetic acid by incubation on ice for 30 min. Precipitates were collected by gel filtration on a column of GF/C filters and counted in scintillation fluid. The amount of receptor synthesized was calculated from the specific activity of the isotope: Hc-erbA contains 12 methionine residues.

**Autin-Biotin Complex DNA Binding Assay—** The procedure of Glass et al. (25) was used to analyze the binding of labeled receptors to DNA fragments. Briefly, complementary oligodeoxyribonucleotides (synthesized using an Applied Biosystems 380A DNA Synthesizer) were designed to have 15-20 base single-stranded overlapping regions which, following annealing, were filled in by incubation with Klenow fragment and 250 μM each of dATP, dCTP, dGTP, and 200 μM of biotin-11-dUTP (Bethesda Research Laboratories). [35S]PITC was included to monitor the fill-in reaction. Double-stranded oligodeoxyribonucleotides representing fragments of the rat a-subunit gene are described under "Results" and the legend to Fig. 3. A double-stranded oligodeoxyribonucleotide encompassing the putative TRE was synthesized using the Sequenase kit (US Biochemicals) and was used as a positive control in the assay. Oligodeoxyribonucleotides corresponding to sequences of the second exon of the rat a-subunit gene (+80 to +142) (29) and the third exon of the chicken actin gene (+2070 to +2146) (38) were included as negative controls. All oligodeoxyribonucleotides were designed to have 11-15 bases complementary to each strand, and all baits were radiolabeled with T3 in an agarose equally well, as assessed by precipitation of p32-labeled DNA in the absence of protein.

**Nuclear extracts in binding buffer were preincubated with [125I]T3 (1 nM), to saturate the receptor with [125I]T3, poly(dI-dC) (250 μg/ml), and Nonidet P-40 (0.1%), before the biotin-labeled DNA (1 pmol) was added, and the incubation was continued for 40 min. Protein-DNA complexes were precipitated by the addition of 20 μl of streptavidin-agarose (BRL; 50% suspension in binding buffer). Pellets were collected by centrifugation and washed three times in the binding buffer containing 0.1% Nonidet P-40. T3 bound to the streptavidin-agarose was determined by radioactivity of the washes. Binding of the Hc-erbA protein to biotinylated oligomers was assessed by incubation of 3-5 μl of 325-labeled protein (2.9 × 10^6 cpm) with biotinylated DNA in 40 μl of binding buffer with poly(dI-dC) (250 μg/ml), Nonidet P-40 (0.1%), and nonradiolabeled T3 (10 nM). After incubation at room temperature for 40 min, protein-DNA complexes were precipitated with streptavidin-agarose as described above. The washed pellets were transferred to scintillation vials with 0.5 ml of water, and 10 μl of Ultrasol (National Diagnostics) was added for scintillation counting.

**RESULTS**

A restriction enzyme map of the 5'-end of the rat a-subunit gene is shown in Fig. 1A. The sequence of this gene has been recently reported (39); a single transcriptional start site was identified, and nucleotides were numbered with the transcriptional start site designated as +1 and negative numbers refer to nucleotides preceding the cap site. A fragment extending from the EcoRI site to -460 site to the XhoII site (+68) site was isolated and subcloned into pUC13CAT. An XhoII site fragment (−170 to +36), a Sau3A-Sau3A fragment (−80 to +33) and a PstI-Sau3A fragment (−4 to +33) were similarly inserted into the BamHI site of the Smal site of the polynucleotide of pUC13CAT to create the various aCAT constructs. The identity and orientation of each of the constructs were verified by restriction mapping and DNA sequencing by the method of Maxam and Gilbert (40). The promoter activity of each of these constructs was measured by transient transfection into GH3 cells in the presence of 10^{-10} and 10^{-9} M T3 (Fig. 1B). It is important to note that GH cells require T3 for growth (23, 41), and in the absence of exogenously added T3, both the growth rate and the transfection efficiency of GH3 cells decline. The addition of 10^{-9} M T3 is required to restore growth and transfection competence in this system although this level of T3 may blunt the apparent response to T3. Deletions
to delineate the 5'-end and either the Sau3A, a-subunit gene. Restriction fragments, using the designated enzymes to delineate the 3'-end, were inserted into the polylinker of a Ta. CAT activity was assayed as described under "Materials and Methods." The transfection results are a composite of two experiments, although the magnitude of the difference varied from experiment to experiment. The apparent decreases and increases could be due to the removal of enhancer and repressor elements, respectively, or could be due to variations in plasmid preparations or transfection efficiencies. Nevertheless, the changes in basal expression are independent of the effect of Ts and expression of the three larger aCAT constructs is suppressed in GHS cells grown for 48 h in the presence of 10⁻⁸ M Ts (Fig. 1B), compared to expression in the presence of 10⁻¹⁰ M Ts. A 40–74% decrease in CAT activity was found following exposure to 10⁻⁸ M Ts with the various constructs. Deletion to 80 bp of 5'-flanking region did not destroy either basal activity or Ts responsiveness. Further deletion or removal of the TATA sequence, as demonstrated by the smallest a-4 construct, decreased activity of the promoter to a point where an effect of Ts could not be detected, suggesting that the sequences in the gene which mediate the effect of thyroid hormone are localized within the 114 bp found in the a-80CAT construct. To control for the effects of Ts on the transient expression system, parallel transfections were performed with the TKCAT plasmid. The expression of TKCAT was unaffected by media containing 10⁻⁸ M Ts, as compared to expression found in the presence of 10⁻¹⁰ M Ts.

To examine the dose dependence of the effect of Ts on the activity of the a-subunit gene promoter, GH3 cells transfected with the a-80 construct were cultured in media containing hypothyroid calf serum supplemented with various concentrations of Ts. As shown in Fig. 2A, CAT expression decreases 33% upon addition of 10⁻⁹ M Ts (compared to activity seen with 10⁻¹⁰ M Ts); 40 and 53% decreases were seen with 10⁻⁸ and 10⁻⁷ M Ts, respectively. The magnitude of the response in this system is consistent with that found in vivo in rat pituitary and in thyrotropic tumors (13, 15). A representative autoradiograph showing the promoter activity of the a-80CAT construct is shown in Fig. 2B. When the DNA fragment is inserted in the 5' to 3' orientation (relative to the orientation of the CAT sequence), it functions as a promoter for the

FIG. 1. A, restriction enzyme map of 5'-flanking region of the rat a-subunit gene. Restriction fragments, using the designated enzymes to delineate the 5'-end and either the Sau3A, MnlI, or the XhoII site to delineate the 3'-end, were inserted into the polylinker of a PUC18CAT plasmid as described under "Materials and Methods." B, effect of Ts on expression of aCAT constructs in GH3 cells. GH3 cells were transfected with 5–10 μg of plasmid DNA and cultured in media containing stripped serum supplemented with 10⁻¹⁰ (M) or 10⁻⁸ M (M) Ts. CAT activity was assayed as described under "Materials and Methods." The transfection results are a composite of two experiments, and the data represent the mean of quadruplicate flasks ± S.E. The asterisks indicate the values from cells incubated with 10⁻⁸ M Ts which are significantly lower than the values from cells incubated with 10⁻¹⁰ M Ts at p < 0.025 (*) and p < 0.0005 (**).

FIG. 2. A, effect of increasing concentrations of Ts on expression of aCAT in GH3 cells grown in media containing 10% hypothyroid serum, supplemented with Ts. GH3 cells were transfected with 5 μg of plasmid DNA and CAT activity was assayed as described under "Materials and Methods." The transfection results represent the mean of quadruplicate flasks ± S.E. ** indicate that the values are significantly lower than the values in the first column (p < 0.05). B, expression of α-80CAT in GH3 cells. Representative autoradiogram of a thin layer chromatographic plate showing acetylation of [³⁵Cl]chloramphenicol by cell extracts prepared from GH3 cells transfected with 5 μg of the following plasmids: 5' → 3' contains a gene sequences from -80 to +33 oriented 5' to 3' relative to the CAT gene; 3' → 5' contains the same fragment in the reverse orientation, and TKCAT contains the herpes simplex virus thymidine kinase promoter.
expression of CAT (Fig. 2B). When placed in the opposite orientation (3' to 5'), no CAT activity was detected. In addition, no CAT activity was detected when cells were transfected with a promoterless plasmid (not shown). This fragment of the α-subunit gene promoter is a relatively weak promoter and appears to be 2-10-fold less active in these cells compared to the herpes simplex virus thymidine kinase promoter (TKCAT).

To investigate the association of the T3R with this region of the rat α-subunit gene, we have used an assay that directly analyzes the binding of the T3R to specific nucleic acid sequences. We have previously used this protocol to demonstrate specific binding of different forms of in vitro synthesized erbA protein to the rGH TRE (37). Double-stranded oligodeoxyribonucleotides spanning portions of the region of the α-subunit gene present in the smallest construct conferring the α-subunit gene present in the smallest construct confer-ving the α-subunit gene present in the smallest construct conferring the α-subunit gene present in the smallest construct conferring the α-subunit gene present in the smallest construct conferring the α-subunit gene present in the smallest construct conferring T3 regulation on CAT expression (α-80CAT) were synthesized with single-stranded ends such that biotin-11-dUTP was incorporated, as described under "Materials and Methods." The oligodeoxyribonucleotides spanning this region are shown in Fig. 3. Oligodeoxyribonucleotides representing the 5'- or 3'-ends of the α-80 to +33 fragment were synthesized with single-stranded ends such that biotin-11-dUTP was incorporated, as described under "Materials and Methods." The oligodeoxyri-bonucleotide representing sequences -74 to -25 of the rat α-subunit gene sequence has also been tested in competition studies with the nonbiotin-labeled rGH fragment versus the nonbiotinylated α-subunit gene fragment. Formation of the nonbiotinylated T3R complex is inhibited 50% by a 2-3-fold molar excess of nonbiotinylated α-74 to -25 oligonucleotide. Formation of the complex is not inhibited by the nonbiotinylated α-exon1 fragment until a 80-100-fold molar excess is present (data not shown).

To define further the sequences involved in binding the receptor the α-74 to -25 oligodeoxyribonucleotide was dis-sected and smaller oligodeoxyribonucleotides tested for their ability to bind the [125I]T3-receptor complex (Fig. 5A). The α-74 to -25 and the α-74 to -38 oligodeoxyribonucleotides showed comparable levels of binding of the T3R; α-52 to -23 did not bind, indicating that the binding region was localized within the α-74 to -38 oligodeoxyribonucleotide. Binding of T3R to the α-subunit gene sequences was comparable to the binding seen with the rGH gene fragment, which is 9-fold higher than the nonspecific binding exhibited by the actin gene fragment (Fig. 5A).

These fragments were also tested for their ability to bind the in vitro translated human c-erbAβ gene product. The binding of the α-subunit gene oligodeoxyribonucleotides and the control oligodeoxyribonucleotides to [35S]methionine-la-bled c-erbAβ are shown in Fig. 5B. Both the α-74 to -25 and the α-74 to -38 oligodeoxyribonucleotides bound the in vitro translation product significantly. As was found with the T3R receptor from nuclear extracts, the α-52 to -23 oligodeoxyribonucleotide did not demonstrate any binding significantly greater than the binding seen without DNA or with the actin gene fragment. Similar binding data were obtained with [125I]T3-bound in vitro translated protein (data not shown). Both the 48-kDa and the 52-kDa translation products bound the DNA as determined by sodium dodecyl sulfate gel electrophoresis of radioactive protein eluted from the strept-avidin-agarose pellet (data not shown). Compared to the binding found with the rGH gene fragment, the α-74 to -25 and α-74 to -38 oligodeoxyribonucleotides bound the in vitro translation product significantly.

Fig. 3. Sequence of oligodeoxyribonucleotide constructs used to measure T3 receptor binding to the rat α-subunit gene. The sequence of the ρα-subunit gene from base -80 to +33 is shown in the top row. The TATA box is underlined. Nucleotides are numbered with the single transcriptional start site (bent arrow) designated as +1; negative numbers refer to nucleotides preceding the start site. Synthetic oligodeoxyribonucleotides spanning this region are shown in the lower four rows. Upper case letters indicate sequences comprising the synthetic oligodeoxyribonucleotides; lower case letters are bases incorporated during the reaction with Klenow. Random sequences designed for the incorporation of biotin-11-dUTP residues (a) are shown outside the boxed regions, which indicate a gene sequence.
progressive decreases of a-subunit gene transcription which were performed with the total trichloroacetic acid-precipitable counts/min, and no activity and the transcriptional response to T3. Based on down-regulation of expression of the transfected aCAT construct; such sequencess may be a silencer region, or may be involved in the higher order structure of the chromosome. Alternatively, absence of modifications such as methylation may permit the utilization of the transfected promoter. Nevertheless, it is conceivable that the GH3 cells do have tissue-specific factors that are also found in pituitary cells that normally express the a-subunit gene. A common pituitary transcriptional activator, Pit 1, has been described for the prolactin and growth hormone gene, and its presence in thyrotropes has been postulated (44). The somatotrophic pituitary cell lines do have a well characterized T3 response (45), and it appears that the endogenous receptor can function to down-regulate the expression of the transfected rat a-subunit gene promoter.

By use of the avidin-biotin complex DNA binding assay we have localized a T3R binding region of the rat a-subunit gene that is contained within the smallest construct that confers T3 responsivity. The a–74 to –38 oligodeoxyribonucleotide binds T3R from GH3 cells and also binds the in vitro translated c-erbAβ gene product. Fragments of the a-subunit gene (exon 2, fragments –52 to –23 and –22 to +33) did not bind either receptor preparation. This indicates that the T3R binds in a sequence-specific manner. Inasmuch as reticulocyte lysate used to synthesize the Hc-erbAβ protein likely lacks any pituitary-specific factors, the receptor probably can bind di-
directly to the α-subunit gene sequences in the absence of such factors. Binding of the T3R to a specific sequence within the range of the TRE is consistent with the possibility that the inhibition of α-subunit gene transcription is mediated by direct binding of T3R to the gene. Transcriptional regulation by direct binding of a hormone receptor to the responsive element of a gene is also observed with other members of the steroid receptor/thyroid hormone receptor superfamily; the Zn2+ fingers formed by multiple cysteine- and histidine-rich repeating units in these proteins are thought to interact with DNA (46).

Compared to the binding seen with the rGH gene fragment, the c-erbAβ protein bound the α-fragments less avidly than the receptor prepared from the GH3 nuclear extracts bound rGH and α-subunits gene fragments equally well. This suggests that GH3 nuclear extracts may contain other factors which enhance the binding of the receptor to the α-subunit gene, and may be involved with suppression of the transcription rate.

The T3R binding sequences in the rGH gene have been mapped by footprinting techniques, and functional elements have been identified using stable and transient expression studies (22–25). The precise location of the TRE is still unclear, and a consensus sequence for thyroid hormone receptor binding has yet to be identified. However, an imperfect direct repeat between –187 and –169 has been identified by Koenig et al. (47), while Glass et al. (25) have localized binding to –179 to –164 using Dnase I footprinting. More recently, Glass et al. (48) have described the binding of the T3R to variations of a palindrome that resemble the vitellogenin A2 estrogen response element. A computer comparison of these regions of the rGH gene with α–74 to 38 of the rat α-subunit gene reveals no sequence similarity. The lack of consensus sequence among target sequences bound by a single receptor is not unique: the yeast HAP1 activator binds to sites that display sequence degeneracy (45). In addition, the expression of the rGH gene is stimulated by T3, and the α-subunit gene expression is suppressed; thus, different sequences may mediate activation and repression. The mechanism by which the same receptor can mediate both positive and negative effects on transcription is unknown. Other proteins may be involved in the effects of T3 on gene expression. Akerblom et al. (50) have recently proposed that the negative regulation of α-subunit gene expression by glucocorticoids occurs through interference with the binding or function of a CAMP response element binding protein. Future studies are aimed at determining the nature of the interaction between the T3R and other transcriptional factors involved in repression of gene expression.

REFERENCES