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Ligand-Dependent, Pit-1/Growth Hormone Factor-1 (GHF-1)-Independent Transcriptional Stimulation of Rat Growth Hormone Gene Expression by Thyroid Hormone Receptors In Vitro

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The expression of the rat growth hormone (rGH) gene in the anterior pituitary gland is modulated by Pit-1/GHF-1, a pituitary-specific transcription factor, and by other more widely distributed factors, such as the thyroid hormone receptors (TRs), Sp1, and the glucocorticoid receptor. Thyroid hormone (T_3)-mediated transcriptional stimulation of rGH gene expression has been extensively studied in vivo and in vitro including the measurements of (i) rGH mRNA by blot hybridization, (ii) transcriptional rate of rGH gene by nuclear run-on, and (iii) reporter gene expression in which a chimeric plasmid containing 5′-flanking sequences of the rGH gene linked to a reporter gene has been transfected either stably or transiently into pituitary and/or nonpituitary cells. From these studies, it has been suggested that the Pit-1/GHF-1 binding site is necessary for full T_3 action. We developed a cell-free in vitro transcription system to examine further the roles of the TRs and Pit-1/GHF-1 in rGH gene activation. Using GH_3 nuclear extract as a source of TRs and Pit-1/GHF-1, this in vitro transcription assay showed that T_3 stimulation of rGH promoter activity is dependent on the addition of T_3 to the GH_3 nuclear extract. This transcriptional stimulation was augmented with increasing concentrations of ligand and was T_3, but not T_4 or reverse T_3, specific. T_3-mediated stimulation of rGH promoter activity was completely abolished by preincubation of the nuclear extract with rGH-thyroid hormone response element (−200 to −160) but not with Pit-1/GHF-1 (−137 to −65) oligonucleotides. Further, neither deletion of both Pit-1/GHF-1 binding sites nor mutation of the proximal Pit-1/GHF-1 binding site from the rGH promoter abrogated the T_3 effect. These results provide evidence that T_3-stimulated rGH promoter activity is independent of Pit-1/GHF-1 and raise the possibility that the stimulation of rGH gene expression by T_3 might involve direct interaction of TRs with the general transcriptional apparatus.

Growth hormone (GH) synthesis and secretion is limited largely to the somatotropic cells of the anterior pituitary gland. Extensive characterization of the promoter region of the rat GH gene has shown that Pit-1/growth hormone factor-1 (GHF-1), a pituitary-specific transcriptional factor, plays a major role in this tissue-specific expression of the GH gene (32). The locations of Pit-1/GHF-1 binding sites in the promoter of the GH gene have been determined by DNase I footprinting. They comprise two adjacent sites, one at −80 (proximal) and the other at −120 (distal), upstream of the start site of transcription of the GH gene (5, 21). Both these sites are required for in vivo and in vitro transcription as well as basal and cell-type specific GH gene expression (28, 32). Furthermore, purified Pit-1/GHF-1 stimulates transcription from the GH promoter when added to nuclear extract of HeLa cells, a tissue that does not express GH or Pit-1/GHF-1 (6, 11).

Thyroid hormone (3,5,3′-triiodo-L-thyronine, T_3) can stimulate rat GH (rGH) gene expression in anterior pituitary cells and in several pituitary tumor cell lines, such as GH_3 and GH_1 cells (16, 36, 37). In GH_1 cells, T_3 treatment results in a 3- to 10-fold increase in GH synthesis which is paralleled by augmented levels of rGH mRNA. Yaffe and Samuels (47) also showed that the transcription rate of the rGH gene is modulated within minutes of T_3 exposure. Furthermore, changes in the rate of transcription of rGH gene parallel the T_3 occupancy of the nuclear receptor. The identification of c-*erbA* gene products as T_3 receptors (TRs) (40, 45) has led to further characterization of TRs and TR-DNA interactions. In a stable and transient transfection study, Glass et al. (19) reported that a T_3 response element (TRE) is localized between −177 and −166 of the rGH promoter (19). Brent et al. (7) have demonstrated that the rGH-TRE (−189 to −167) consists of three hexamers, with TRE half-site consensus sequences, AGGT(C/A)A, arranged as a direct repeat (AB domains) and as an overlapping inverted palindromic domain (BC domains). These are required for full T_3-mediated transcriptional activation (7). However, the TRE identified by the internal deletion mutant study of Koenig et al. (25) suggests that a functionally important region resides between −190 and −172. Ye et al. (48) found that sequences between −236 and −146 of the rGH promoter alone did not confer T_3 regulation to either enhancerless simian virus 40 or enhancerless Rous sarcoma viral promoters. However, regulated expression of these heterologous promoters occurred if sequences containing the cell-specific elements (Pit-1/GHF-1 binding sites) were ligated to the foreign promoter along with the upstream rGH-TRE. They proposed that both cell-specific elements and rGH-TRE are required for T_3 stimulation of rGH gene expression, a conclusion that contrasts with the results of Brent et al. (8), which show that the rGH-TRE alone placed in front of thymidine kinase promoter can mediate a T_3 response. Recently, Schaufele et al. (41) reported that, in the presence of protein kinase A and/or

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C stimulators (either forskolin or phorbol 12-myristate 13-acetate), there was a synergistic activation of the rGH promoter activity by coexpression of Pit-1/GHF-1 and TRs in human monocyte U937 cells. They postulated that this kinase-mediated phosphorylation event may be important to both the TR and Pit-1/GHF-1 activation pathways. However, the precise location of the critical *cis*-acting sequences of the rGH gene and the roles of Pit-1/GHF-1 and TRs in T_3-regulated rGH gene expression remain to be determined. The reason for this discrepancy is not clear, but could involve differences in vectors, heterologous promoters, cell lines, and/or transfection protocols.

Cell-free in vitro transcription is an useful functional assay to study the interaction of different transcriptional factors that cooperate to control gene expression. Precise dissection of steroid hormone receptor-mediated transcription at the molecular level has successfully been performed in functional cell-free systems (2, 12, 18, 23). These studies have confirmed that nuclear receptors act as sequence-specific transcriptional factors, and the technique provides a valuable approach for examining the protein-protein interactions that are required by nuclear receptor-mediated gene expression. In this study, we aimed to determine the roles of TRs and Pit-1/GHF-1 in rGH gene activation using a cell-free in vitro transcription system, and we present evidence that T_3 stimulation of rGH promoter activity is T_3, but not Pit-1/GHF-1, dependent.

MATERIALS AND METHODS

Oligonucleotides and plasmids. Plasmids used in the in vitro transcription assays were constructed by standard procedures (35). Briefly, rGH250/G and rGH150/G plasmids were constructed by insertion of polymerase chain reaction-generated DNA fragments (−250 to −1 and −150 to −1, respectively) from the rGH promoter into *Bgl*II-*Sac*I sites of pLovTATA plasmid in which a 380-bp G-free cassette is located adjacent to the *Sac*I site (23). rGH60/G plasmid was prepared by subcloning a synthetic oligonucleotide which spans −60 to −1 of the rGH promoter into the *Bgl*II-*Sac*I sites of the pLovTATA plasmid. rGHTRE/G plasmid was made by introduction of a synthetic rGH-TRE (−200 to −160 of the rGH promoter) oligonucleotide into the *Bgl*II site of the rGH60/G plasmid. rGH250m/G was constructed by polymerase chain reaction-based mutagenesis in which ATAAAT (−84 to −79) (within the downstream Pit-1/GHF-1 site) was changed to TCTAGA in the rGH promoter and subcloned into pLovTATA plasmid. AdML/G plasmid is a derivative of pMLC2AT19 and contains the adenovirus major late (AdML) promoter (−400 to +10) linked to a G-free cassette of 200 bp. This plasmid served as an internal control in all of the in vitro transcription assays. All plasmids were purified by banding twice with ethidium bromide-CsCl centrifugation. DNA concentrations were determined by spectrophotometry and verified by agarose gel electrophoresis. The sense strands of oligonucleotides (5′ to 3′) used in the electrophoretic mobility shift assay (EMSA) and in vitro transcription assay are shown as follows, and the consensus sequences which correspond to TRE half-site and Pit-1/GHF-1 binding site are underlined. rGH-TRE −200 to −160: GATCCGGCGGTGGAAAGGTAAGATCAGGGACGTGACCGCAGGA; mutated rGH-TRE: GATCCGGCGGTGGAAAGGTAAGATCATTGACGTGACCGCAGGA; Pit-1/GHF-1 binding sites −137 to −65: GGGAGGAGCTTCTAAATTATCCATCAGCACAAGCTGTCAGTGGCTCCAGCCATGAATAAATGTATAGGGAAA; mutated Pit-1/GHF-1 binding sites: GGGAGGAGCTTCTAAATTATCCATCAGCACAAGCTGTCAGTGGCTCCAGCCATGATCTAGAGTATAGGGAAA.

Cell culture and preparation of nuclear extracts. Nuclear transcription extracts were prepared as described by Dignam et al. (15). GH_3 and HeLa cells were grown in Spinner flasks in Joklik's modified minimum essential medium (GIBCO, Grand Island, N.Y.) with 12.5% horse serum and 2.5% fetal calf serum, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. When the cell density reached 8×10^5/ml, the medium was changed to Joklik's modified minimum essential medium with 6% horse serum depleted of T_3, as described by Raaka and Samuels (34). After 24 h of incubation, cells were harvested by centrifugation and washed in ice-cold phosphate-buffered saline. The cell pellets were resuspended in 4 original cell volumes of hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 1 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 2 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride) and swollen in ice for 10 min. The cell suspension was then centrifuged (400 × *g*, 10 min, 4°C) and resuspended in 2 original cell volumes of hypotonic buffer plus protease inhibitor mix (2 mM phenylmethylsulfonyl fluoride, 10 mg of leupeptin per ml, 5 mg of aprotinin per ml). The cells were allowed to swell for an additional 5 min and then disrupted with an iced Dounce glass homogenizer (Wheaton) (B-size) to prepare the nuclei. After 20 strokes, the preparation was examined under a phase-contrast microscope, and more strokes applied until 75 to 85% of the cells were broken. The preparation was centrifuged (15,000 × *g*, 10 min, 2°C), and the supernatant was removed. The nuclei were resuspended in 1 nuclear pellet volume of extraction buffer (20 mM HEPES [pH 7.9], 25% glycerol, 0.5 M KCl, 3 mM $MgCl_2$, 0.75 mM spermidine, 0.15 mM spermine, 0.5 mM EDTA, 3 mM DTT, protease inhibitor mix). The preparation was stirred slowly in an ice bath, and the extraction was continued for 40 min. The preparation was centrifuged (20,000 × *g*, 20 min, 2°C), and the supernatant was collected and dialyzed against two changes of nuclear dialysis buffer (20 mM HEPES [pH 7.9], 20% glycerol, 60 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride) for 2 h per change, and the precipitate was removed by centrifugation (15,000 × *g*, 10 min, 4°C). The protein concentration of the supernatant was determined, and the nuclear extract was aliquoted and rapidly frozen and stored in liquid nitrogen.

EMSA. In the standard EMSA, DNA fragments were end labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Gel-purified probes (0.5 ng) were incubated with GH_3 nuclear extract (4 to 5 mg/ml) on ice for 30 min in the presence of 20 mM HEPES (pH 7.9), 60 mM KCl, 2 mM DTT, 5% glycerol, 1 mg of bovine serum albumin per ml, 5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 4 μg of poly(dI/dC), and 2 μg of sonicated salmon sperm DNA. In cold competition experiments, various unlabeled DNA fragments were incubated with the GH_3 nuclear extract for 20 min, and then labeled DNA probe (0.5 ng) was added. Reaction mixtures were subjected to electrophoresis through a 5% polyacrylamide gel (29:1 acrylamide-bisacrylamide) in 0.5× Tris-borate–EDTA buffer for 90 min at 4°C; the gel was dried and then subjected to autoradiography.

Binding of ^{125}I-T_3 to TR. T_3 binding was performed by the procedure of Lazar et al. (27). Briefly, 10 μl of GH_3 nuclear extract (4 to 5 mg/ml) was incubated for 18 h at 4°C with various concentrations of ^{125}I-T_3 (2,200 Ci/mmol; Dupont,

NEN Research Products) in a total volume of 200 μl of KMTD (0.3 M KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.0], 1 mM DTT). Dowex AG-1x8 resin was used to separate bound and free ligand, and nonspecific binding was measured in the presence of a 600-fold molar excess of unlabeled ligand. For competition experiments, 0.5 nM ^{125}I-T_3 was mixed with various concentrations of the appropriate analogs, and binding was analyzed above.

Cell-free in vitro transcription assay. A typical transcription assay included test template (e.g., rGH250/G), internal control template (AdML/G), transcription reaction mixture, and GH_3 nuclear extract in the presence or absence of T_3. The reaction was initiated by the addition of nucleotides and continued for 60 min at 30°C. Unless stated otherwise, a standard reaction mixture (30 μl) contained 20 mM HEPES (pH 7.9), 60 mM NaCl, 6 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, 15% glycerol, 0.5 mM ATP, 0.5 mM CTP, 20 μM unlabeled UTP, 25 μCi of [α-^{32}P]UTP, 1 mM 3′-*O*-methyl GTP, 5 mM creatine phosphate, 20 U of RNase T_1, 0.5 μg of sonicated salmon sperm DNA, 14 μl of GH_3 nuclear extract (4 to 5 mg/ml), 100 ng of internal control template (AdML/G), 100 ng of the test template, and T_3 in indicated amounts. After incubation, the reactions were terminated by adding 70 μl of stop mixture (25 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.5% sodium dodecyl sulfate, 200 μg of *Saccharomyces cerevisiae* tRNA per ml, 400 μg of proteinase K per ml) and incubated for 30 min at 37°C. After the addition of 200 μl of 7 M urea (in 10 mM Tris-HCl–1 mM EDTA [pH 8.0]), each reaction mixture was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated, and subjected to electrophoresis through a 6% polyacrylamide–7 M urea sequencing gel. Autoradiography of dried gels was performed at −80°C with intensifying screens. A typical exposure time was 36 h.

RESULTS

Cell-type specific promoter activity and role of Pit-1/GHF-1 binding sites in basal transcription of rGH promoter. We analyzed the ability of different regions of the rGH promoter to direct basal transcription in an in vitro transcription system using either GH_3 or HeLa cell nuclear extracts. First, we linked various portions of the 5′-flanking region of the rGH gene in front of a 380-bp G-free cassette (Fig. 1A). A template containing the adenovirus major late promoter (AdML/G) (−400 to +10) linked to a shorter G-free cassette (200 bp) was included in the reaction mixture as an internal control. Figure 1B shows that two different test templates, one containing Pit-1/GHF-1 binding sites (rGH150/G) and the other containing an additional TRE (rGH250/G), gave the same basal transcription without adding T_3 to GH_3 nuclear extracts (lanes 1 and 2). When the two Pit-1/GHF-1 binding sites were deleted, the basal transcription of the core promoter (rGH60/G) was decreased to 30% of the basal transcription of the template containing two Pit-1/GHF-1 binding sites (rGH150/G) (Fig. 1B, compare lanes 2 and 3). Disruption of the Pit-1/GHF-1 high-affinity binding site in the rGH promoter also caused a significant decrease of the basal transcription (see Fig. 6; compare lanes 1 and 3). We then compared the basal transcription of the rGH250/G template in GH_3 and HeLa cell nuclear extracts. No detectable basal transcription from rGH250/G was found in the HeLa nuclear extract, but transcription from the internal control template (AdML/G) in the HeLa nuclear extract was sixfold higher than that observed in the GH_3 nuclear extract (Fig. 1B, lanes 1 and 4). In other experiments, α-amanitin (1 μg/ml) completely blocked the basal transcription activities of both test and internal templates when it was added to the transcription reaction mixture (data not shown). These results confirm that (i) the rGH promoter is active in rat pituitary GH_3, but not in HeLa, cell nuclear extracts, and (ii) Pit-1/GHF-1 binding sites are required for the high-level transcription from the rGH promoter.

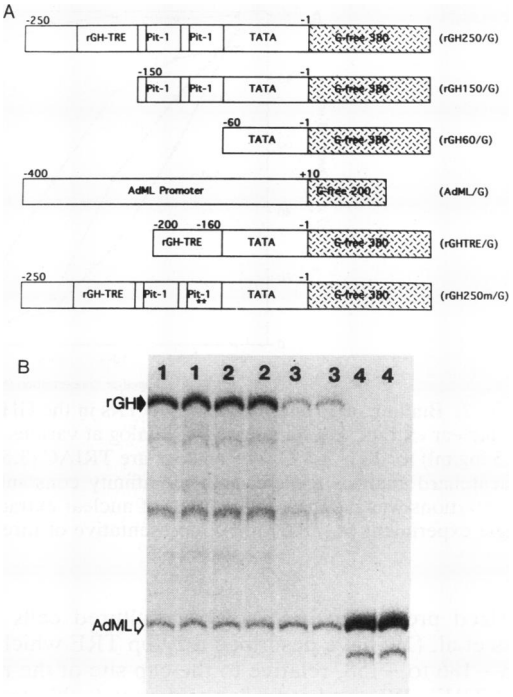

FIG. 1. DNA templates and in vitro transcription of rGH promoter in GH_3 and HeLa nuclear extracts. (A) Schematic diagram of the DNA templates used in the in vitro transcription experiments. rGH250/G, rGH150/G, and rGH60/G plasmids were prepared by ligating various portions of the rGH promoter in front of a 380-nucleotide G-less cassette. AdML/G, which contains the adenovirus major late promoter (−400 to +10) in front of a 200-nucleotide G-less cassette, was used as a noninducible internal control. In the rGHTRE/G and rGH250m/G plasmids, a deletion of both Pit-1/GHF-1 binding sites (−159 to −61) and a mutation in the proximal Pit-1/GHF-1 binding site (−84 to −79, ATAAAT to TCTAGA), respectively, of the rGH promoter were separately linked in front of a 380-nucleotide G-less cassette. (B) Promoter- and cell-type-specific in vitro transcription. Cell-free in vitro transcription assays were performed as described in Materials and Methods. Typically, 100 ng of rGH250/G (lanes 1 and 4), rGH150/G (lane 2), and rGH60/G (lane 3) and 100 ng of AdML/G were included in each reaction mixture and were incubated with either 14 μl of GH_3 (4 to 5 mg/ml) (lanes 1 to 3) or 9 μl of HeLa (7 to 8 mg/ml) (lane 4) nuclear extract. Each reaction was performed in duplicate. The filled and open arrowheads indicate correctly initiated transcripts from the test and the internal control (AdML/G) templates, respectively.

Authentic TRs and Pit-1/GHF-1 protein are present in GH_3 nuclear extract. To investigate whether GH_3 nuclear extract contains authentic TRs and Pit-1/GHF-1 protein, we performed ^{125}I-T_3 ligand binding assay and EMSA. Analysis of the binding data obtained under equilibrium conditions indicated that ^{125}I-T_3 binds to endogenous TRs with a K_d of 0.26 nM (Fig. 2A). The rank order of the competitive binding of T_3 and several T_3 analogs was 3,5,3′-triiodothyroacetic acid (TRIAC) > L-T_3 > L-T_4 > reverse T_3 (rT_3) (Fig. 2B), which is identical to that described for T_3 nuclear receptors char-

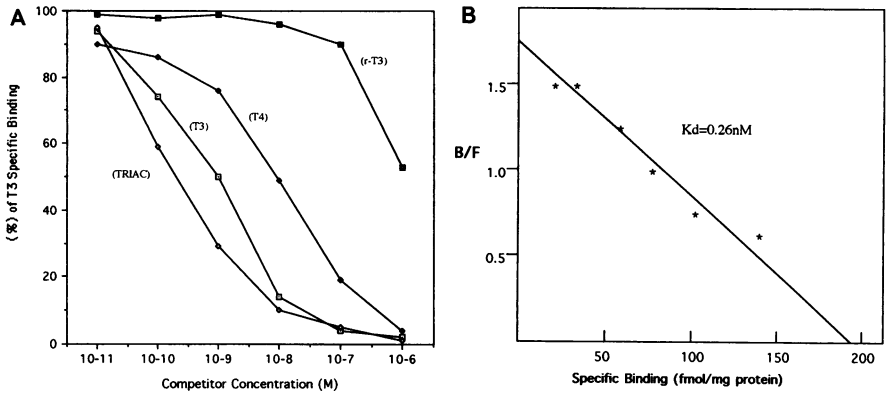

FIG. 2. Binding of T_3 and its analogs to TRs in the GH_3 nuclear extract. (A) Competition of T_3 analogs for binding of ^{125}I-T_3 to TRs in the GH_3 nuclear extract. Each indicated T_3 analog at various concentrations was incubated with 0.5 nM ^{125}I-T_3 and 10 μl of GH_3 nuclear extract (4 to 5 mg/ml) for 18 h at 4°C. The analogs are TRIAC (3,5,3'-triiodothyroacetic acid); T_4 (L-thyroxine); and rT3 (3,3',5'-triiodo-L-thyronine). (B) Scatchard analysis to determine the affinity constant of T_3 binding of salt-extractable TRs in GH_3 nuclear extract. ^{125}I-T_3 at various concentrations was incubated with 10 μl of nuclear extract as described in Materials and Methods. The Scatchard plot depicts the results of a single experiment (K_d, 0.26 nM), representative of three separate binding studies.

acterized previously in tissues or cultured cells (38, 42). Glass et al. (19) have described a 29-bp TRE which extends from −186 to −158, relative to the cap site of the rGH gene (rGH-TRE). We used a similar sequence (−200 to −160) to test the DNA binding activity of TRs in the GH_3 nuclear extract. When the ^{32}P-labeled rGH-TRE (0.5 ng) was incubated with increasing amounts of GH_3 nuclear extract, two specific protein-DNA complexes were formed (Fig. 3, lanes 8 to 10). A 50-fold molar excess of unlabeled rGH-TRE (−200 to −160) abolished the formation of these two complexes (Fig. 3, lane 11); however, the addition of a 50-fold molar excess of unlabeled Pit-1/GHF-1 binding site oligonucleotides (Fig. 3, lane 12) did not decrease the amount of these two DNA-protein complexes. When ^{32}P-labeled mutated rGH-TRE, in which the AGGGAC of B half-site of rGH-TRE was replaced by ATTGAC, was tested, no specific receptor-DNA complexes were formed (data not shown). The DNA binding specificity of Pit-1/GHF-1 in GH_3 nuclear extract was also examined. The oligonucleotide containing both Pit-1/GHF-1 binding sites (−137 to −65) in the rGH promoter was used. When increasing amounts of nuclear proteins (2.5 to 10 μg) were used, the formation of a second complex was observed (Fig. 3, lanes 1 to 3). Both complexes were markedly diminished in the presence of increasing amounts of unlabeled Pit-1/GHF-1 oligonucleotides (Fig. 3, lanes 4 to 6). In contrast, a 50-fold molar excess of unlabeled rGH-TRE oligonucleotide did not affect these two complexes (Fig. 3, lane 7). ^{32}P-labeled Pit-1/GHF-1 oligonucleotide, in which the proximal Pit-1/GHF-1 binding site (−84 to −79) was mutated, disrupted the formation of both complexes even in the presence of 10 μg of GH_3 nuclear extract (data not shown). These results indicate that endogenous TRs in the GH_3 nuclear extract retain both ligand and DNA binding affinity and specificity. Furthermore, endogenous Pit-1/GHF-1 also retains specific, high-affinity DNA binding to its cognate DNA.

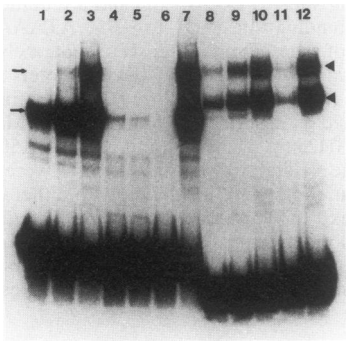

FIG. 3. DNA binding properties of Pit-1/GHF-1 and TRs in the GH_3 nuclear extract. Double-stranded oligonucleotides used for Pit-1/GHF-1 binding contained two Pit-1/GHF-1 binding sites (−139 to −66), and those used for TR binding contained rGH-TRE (−200 to −160) and were end labeled with T_4 polynucleotide kinase. GH_3 nuclear extracts (4 to 5 mg/ml) (0.5 μl [lane 1], 1 μl [lane 2], and 2 μl [lanes 3 to 7]) were incubated with ^{32}P-labeled Pit-1/GHF-1 oligonucleotide. For the competition study, a 5-fold (lane 4), 10-fold (lane 5), and 20-fold (lane 6) molar excess of unlabeled Pit-1/GHF-1 oligonucleotide and a 50-fold molar excess (lane 7) of unlabeled rGH-TRE oligonucleotides were preincubated with 2 μl of GH_3 nuclear extract. DNA binding activity of the endogenous TR was determined in a similar manner. ^{32}P-labeled rGH-TRE oligonucleotide was mixed with 0.5 μl (lane 8), 1 μl (lane 9), and 2 μl (lanes 10 to 12) of GH_3 nuclear extract. Unlabeled rGH-TRE (25-fold [lane 11]) and unlabeled Pit-1/GHF-1 (50-fold [lane 12]) oligonucleotides were preincubated with 2 μl of GH_3 nuclear extract. Specific Pit-1/GHF-1–DNA and TR-DNA complexes are indicated by solid arrows and arrowheads, respectively.

Stimulation of rGH promoter activity by TR is ligand dependent. In the absence of T_3, transcription from the rGH250/G template appears to be the same as that from the rGH150/G template (Fig. 1B, compare lanes 1 and 2). We then investigated the effect of T_3 and its analogs on the stimulation of rGH promoter activity in a standard in vitro transcription reaction (Fig. 4) by incubating T_3 or T_3 analogs (T_4 and rT_3) with GH_3 nuclear extract for 10 min prior to the addition of either the rGH250/G or rGH150/G template. Transcription from the rGH250/G template was increased 4.5-fold (quantified by densitometry) by 10^{-8} M T_3 (Fig. 4, lane 2), but not with 10^{-8} M T_4 (lane 3) or rT_3 (lane 4). When the rGH150/G template was used, which does not contain the rGH-TRE, no transcriptional stimulation was observed with 10^{-8} M T_3 (Fig. 4, compare lanes 5 and 6). We also tested the effect of T_3 on HeLa cell nuclear extract which does not contain endogenous TRs and found that neither the

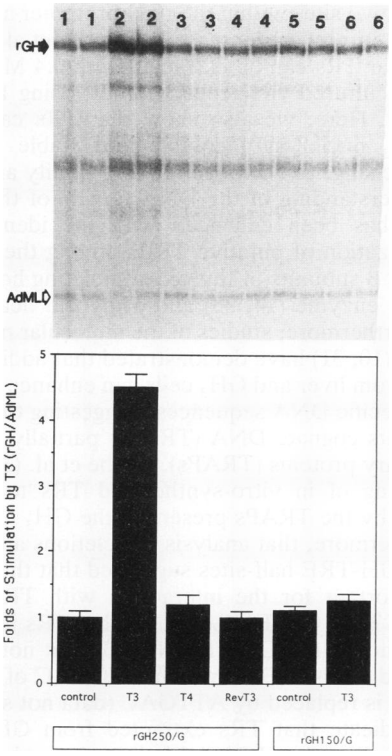

FIG. 4. Specificity of T_3 in T_3-activated transcriptional enhancement of rGH promoter. GH_3 nuclear extracts (4 to 5 mg/ml) were not treated (lane 1) or were treated with 10^{-8} M L-T_3 (lane 2), L-T_4 (lane 3), and rT_3 (lane 4). rGH250/G template was added to the transcriptional mixture as described in Materials and Methods. In lanes 5 (control) and 6 (10^{-8} M T_3), rGH150/G template, which does not contain the rGH-TRE, was used. Reactions were performed in duplicate. Transcription levels were quantitated by densitometry and are presented in bar graph form.

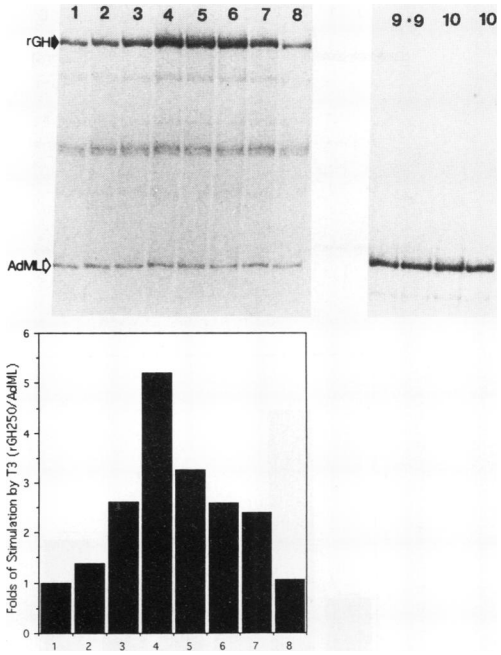

FIG. 5. In vitro stimulation of rGH promoter activity by T_3 is dose dependent. Left panel: in vitro transcription was done without (lane 1) or with 10^{-10} M (lane 2), 10^{-9} M (lane 3), and 10^{-8} M (lanes 4 to 8) T_3 and 14 μl of GH_3 nuclear extract (4 to 5 mg/ml) for 10 min. rGH250/G template (100 ng) was added, and reactions were kept at 30°C for another 60 min. After phenol-chloroform extraction and ethanol precipitation, RNA transcripts were subjected to electrophoresis on 6% polyacrylamide–7 M urea sequencing gels. For the competition study, 20-fold (lane 5) and 50-fold (lane 6) molar excesses of Pit-1/GHF-1 oligonucleotide and 10-fold (lane 7) and 25-fold (lane 8) molar excesses of rGH-TRE oligonucleotide were incubated with GH_3 nuclear extract before the addition of 10^{-8} M T_3. For the right panel, rGH150/G template was used and reactions were performed in duplicate and the same amounts of HeLa nuclear extracts (7 to 8 mg/ml) were incubated without (lanes 9) or with (lanes 10) 10^{-8} M T_3. Transcription levels were quantitated by densitometry and are presented in bar graph form. Data from three independent experiments showed significant T_3-mediated stimulation.

rGH250/G template nor the AdML/G template were stimulated by 10^{-8} M T_3 (Fig. 5, compare lanes 9 and 10). This result indicates that stimulation of rGH promoter activity is ligand (T_3) specific and DNA dependent.

Stimulation of rGH promoter activity by T_3 is concentration dependent and is inhibited by rGH-TRE oligonucleotides. To examine further the specificity of T_3-mediated transcriptional stimulation, GH_3 nuclear extract was incubated with increasing concentrations of T_3 (10^{-10} to 10^{-8} M), and transcription from the rGH250/G template was assessed by densitometry. Maximal transcriptional stimulation (4.2-fold) was achieved at 10^{-8} M T_3 (Fig. 5, lanes 1 to 4). When GH_3 nuclear extract was preincubated with a 20-fold (Fig. 5, lane 5) or 50-fold (lane 6) molar excess of unlabeled Pit-1/GHF-1 oligonucleotide, T_3 (10^{-8} M) gave a moderate but not maximal stimulation of transcription. The decrease in the maximal stimulation of transcription is due to decreased basal transcription level (see below in Fig. 6, compare lanes 1 and 3) rather than insufficient blockade of transcription by unlabeled Pit-1/GHF-1 oligonucleotides, inasmuch as a 20-fold molar excess of unlabeled Pit-1/GHF-1 oligonucleotide completely prevented putative Pit-1/GHF-1 protein-DNA complex formation in the EMSA (Fig. 3, lane 6). On the other hand, T_3-stimulated transcripts were reduced to the basal level by a 25-fold molar excess of unlabeled authentic rGH-TRE oligonucleotide (Fig. 4, lane 8). In a separate experiment, preincubation of 30-fold molar excess of estrogen response element with GH_3 nuclear extract did not block T_3-stimulated rGH promoter activity (data not shown). These result suggests that Pit-1/GHF-1 is essential for basal-level transcription but may not be necessary for T_3-stimulated rGH gene expression.

Pit-1/GHF-1-independent stimulation of rGH promoter activity by T_3. To test directly the role of Pit-1/GHF-1 in T_3-stimulated rGH transcription, we either deleted both Pit-1/GHF-1 binding sites or mutated the proximal Pit-1/GHF-1 binding site from the rGH promoter. The wild-type template rGH250/G gave a fourfold transcriptional stimulation (Fig. 6, lanes 1 and 2). When the high-affinity proximal Pit-1/GHF-1 binding site was mutated (rGH250M/G), which completely abolished the formation of Pit-1/GHF-1-DNA complexes in the EMSA (data not shown), the basal transcription was decreased to 40% of that of the wild-type template (Fig. 6, lanes 1 and 3). However, addition of T_3 (10^{-8} M) still resulted in a fourfold transcriptional stimulation (Fig. 6, compare lanes 3 and 4). After deletion of both Pit-1/GHF-1 binding sites from the wild-type template, basal transcription from the rGHTRE/G template (Fig. 6, lane 5)

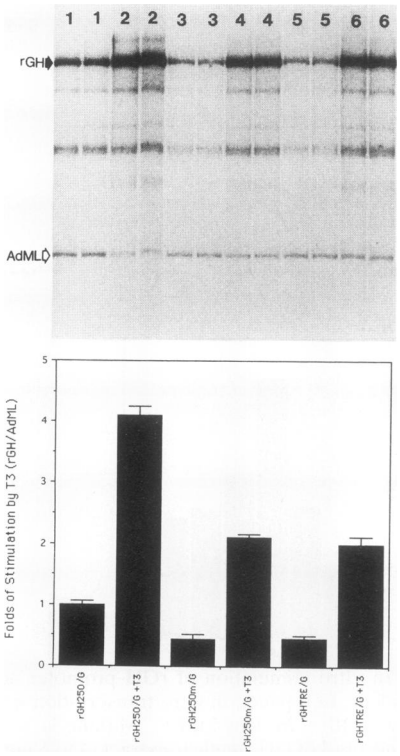

FIG. 6. Ligand-stimulated transcription in vitro is Pit-1/GHF-1 independent. Transcription reactions were the same as previously described. Each reaction was performed in duplicate. rGH250/G template was used in lanes 1 (control) and in lanes 2 (10^{-8} M T_3). rGHTRE/G template, in which both Pit-1/GHF-1 binding sites are deleted, was used in lanes 3 (control) and in lanes 4 (10^{-8} M T_3). rGH250m/G template, which contains a mutated proximal Pit-1/GHF-1 binding site, was used in lanes 5 (control) and lanes 6 (10^{-8} M T_3). Transcripts were quantitated by densitometry and are presented in bar graph form. Data from three independent experiments showed similar levels of T_3 stimulation.

was similar to that from the rGH250m/G template (lane 3). Importantly, transcription from this template was also increased fourfold by T_3 (10^{-8} M) (Fig. 6, compare lanes 5 and 6). Taken together, these data strongly indicate that T_3-stimulated rGH gene expression is independent of Pit-1/GHF-1. Nevertheless, it is possible that Pit-1/GHF-1 might have a non-DNA binding role in T_3-regulated rGH gene expression. However, there presently are no data in support of this view.

DISCUSSION

Using an in vitro transcription assay, we showed that the rGH promoter template containing both Pit-1/GHF-1 binding sites (rGH150/G) supports cell-specific basal transcription. This result agrees with the studies of Flug et al. (17) and Nelson et al. (32) in which the sequence within the first 145 bp of 5′-flanking DNA of the rGH gene is shown to be important in mediating cell-specific basal expression. Previous studies (48) have demonstrated that Pit-1/GHF-1 interacts with two Pit-1/GHF-1 binding sites in the rGH promoter; a proximal site (−95 to −65) exhibits at least 10-fold-higher affinity for Pit-1/GHF-1 than the distal site (−137 to −107). Using the EMSA, we demonstrated that Pit-1/GHF-1 derived from GH_3 nuclear extract binds with increasing affinity to two sites within the rGH promoter as the amounts of nuclear extract are increased. Samuels et al. (39) reported that nuclear TRs can be extracted with 0.4 M KCl from rat liver and cultured GH_1 cells without losing ligand binding specificity. Here, we also show that TRs can be isolated from GH_3 nuclei in a soluble and stable form without apparent change in hormone binding affinity and specificity.

An understanding of the DNA targets of the various TR isoforms has been enhanced with the identification and characterization of putative TREs among the rGH (19, 25, 26), α and β subunits of thyroid-stimulating hormone (9, 13, 46), malic enzyme (14, 33) and α-myosin heavy-chain (22) genes. Furthermore, studies of the molecular mechanisms of T_3 action (10, 31) have demonstrated that additional nuclear proteins from liver and GH_3 cells can enhance the binding of TRs to specific DNA sequences, suggesting that the binding of TR to its cognate DNA (TRE) is partially dependent on TR auxiliary proteins (TRAPs). Beebe et al. (3) also showed that binding of in vitro-synthesized TRs to rGH-TRE is enhanced by the TRAPs present in the GH_3 nuclear extract and, furthermore, that analysis of deletions and point mutations of rGH-TRE half-sites suggested that the B half-site is more important for the interaction with TRAPs than for binding of TRs. Our EMSAs show that TRs in GH_3 nuclear extract bind to the rGH-TRE (Fig. 3), but not to a mutated ^{32}P-labeled rGH-TRE, in which AGGGAC of B half-site of rGH-TRE is replaced by ATTGAC (data not shown). These results indicate that TRs extracted from GH_3 cell nuclei retain their ligand and DNA binding properties.

The TRs are ligand-dependent nuclear transcription factors, and regulation of T_3-responsive genes occurs fully or in part at the transcriptional level. For example, the transcription rates of the rGH (47) and the β subunit of thyrotropin (43) genes are modulated within an hour of T_3 exposure. Our data demonstrate that transcriptional stimulation from the rGH250/G template is concentration dependent and T_3 specific and that there is no T_3-mediated transcriptional stimulation from the rGH150/G template. This is the first time that activation of rGH promoter in vitro has been shown to be both ligand (T_3) and DNA sequence (rGH-TRE) dependent. Ye et al. (48) reported that both cell-specific elements (Pit-1/GHF-1) and TREs were required for T_3-regulated rGH gene expression in a transient transfection assay. In our in vitro transcription assay, preincubation of GH_3 nuclear extract with a 50-fold molar excess of Pit-1 oligonucleotide, which completely abolished Pit-1/GHF-1 DNA complexes in EMSA, did not block T_3-mediated transcriptional stimulation. This suggests that Pit-1/GHF-1 may not play a significant role in the T_3-dependent transcriptional stimulation of rGH gene expression in a cell-free in vitro transcription system. Nevertheless, it is possible that Pit-1/GHF-1 might have a non-DNA binding role in some of the T_3-mediated actions. In further examining this notion, we also found that neither deletion of both Pit-1/GHF-1 binding sites (rGHTRE/G) nor mutation of the proximal Pit-1/GHF-1 binding site (rGH250m/G) from the rGH promoter reduces the transcriptional stimulation by T_3 (10^{-8} M) (Fig. 6). The difference between our data and those of Ye et al. (48) may relate either to their use of a heterologous promoter system in which the rGH promoter (−236 to −47) was placed in front of an enhancerless Rous sarcoma virus core promoter or to the effect of deletion of Pit-1/GHF-1 sites from the rGH promoter that was greater in the in vivo transfection study such that the T_3-induced level of rGH gene expression in vivo is not detectable.

The precise mechanism of transcriptional activation by

upstream DNA binding proteins (transcriptional activators) is still unclear. Several lines of evidence (see reference 30 for a review) show that these activators interact either directly with the RNA polymerase II transcriptional machinery or indirectly through novel proteins (such as adaptors, coactivators, and accessory proteins) to activate their target genes. For example, an acidic transcriptional activation domain of VP16 binds strongly to the human and yeast TATA-binding factors (44). Yet VP16 also binds TFIIB in an interaction that appears to be important for VP16 activation as well (29). Allan et al. (1) also examined the cooperative transcriptional stimulation between steroid hormone receptors and proximal promoter binding factors. Maximal induction of transcription from the mouse mammary tumor virus promoter by glucocorticoid receptor is dependent on the presence of nuclear factor 1 in the transcription reaction. In contrast, effective activation by the glucocorticoid receptor or the progesterone receptor on a glucocorticoid/progesterone response element-linked ovalbumin promoter does not require interaction with the chicken ovalbumin upstream promoter transcription factor in the ovalbumin promoter. The mechanisms of how TRs interact with the transcriptional machinery and then stimulate target gene expression are poorly understood. Recently, however, several groups have shown that retinoid X receptors (RXRα and -β) are TRAPs that enhance DNA binding of TRs (24, 50, 51). RXRα and -β not only enhance TR binding to DNA but also augment the transcriptional activation of TRs on TREs. Yen et al. (49) also reported that T_3 decreased binding of in vitro-translated TR homodimers but not TR-TRAP heterodimers to TREs, a finding which suggests that heterodimers may be the functionally relevant receptor form in T_3-mediated regulation of transcription.

Recent experiments have demonstrated that recombinant human TATA box binding protein and retinoic acid receptor function cooperatively in the transactivation of the retinoic acid receptor β_2 promoter in EC cells in a strictly retinoic acid-dependent manner (4). Ing et al. (20) also showed specific protein-protein interactions between recombinant TFIIB and three members of the steroid hormone receptor superfamily, estrogen receptor, progesterone receptor, and chicken ovalbumin upstream promoter transcription factor, which suggested that these receptors facilitate the transcription of activated genes at least in part via interaction with the general transcription factor, TFIIB. These results provide an example of cooperativity between members of steroid receptor-TR superfamily and the general transcriptional apparatus. The experiments presented herein demonstrate that ligand (T_3) itself can activate rGH gene expression in GH_3 nuclear extract, without the necessity of providing the TRs exogenously, because TRs are normally located in the nucleus. This is different from steroid hormone receptor-mediated gene activation in other cell-free systems in which exogenous receptors need to be added. It also clearly shows that T_3-mediated transcriptional stimulation of rGH promoter activity in a cell-free in vitro transcription system is independent of Pit-1/GHF-1 binding sites. This observation permits the following model regarding the molecular mechanism of T_3-induced transcription of rGH gene expression (Fig. 7). After ligand (T_3) interacts with its nuclear receptor, TR-TRAP complexes might either induce a conformational change providing an interface for direct interaction with the general transcriptional machinery or form additional complexes with basal transcriptional factors in order to facilitate the initiation of transcription. Additional work needs to be performed to strengthen further this notion. The recent cloning of several TRAPs and general transcriptional factors ensures that the cell-free in vitro transcription system will likely provide an invaluable tool for the precise dissection of the protein-protein interactions involved in T_3-regulated gene expression.

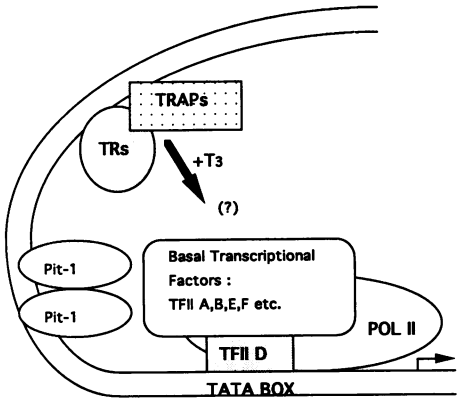

FIG. 7. Model of the transcriptional regulation of rGH gene expression by TRs that is consistent with the results presented in this study. Pit-1/GHF-1 supports cell-type-specific basal, but not T_3-stimulated, transcription of rGH gene expression in GH_3 cells. After T_3 binds to nuclear TRs, we propose that the TRs form heterodimers by their association with TRAPs such as RXRα and -β, etc. The formation of TR-TRAP-T_3 complexes may generate either an activation surface for direct interaction with the general transcriptional machinery or a larger stable complex with basal transcriptional factors which can facilitate the initiation of transcription.

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