cis-Acting Elements of the Rat Growth Hormone Gene Which Mediate Basal and Regulated Expression by Thyroid Hormone*

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In GC cells, a growth hormone-producing rat pituitary cell line, 3,5,3'-triiodo-L-thyronine (L-T3) rapidly stimulates the transcription rate of the growth hormone gene which parallels the level of chromatin-associated L-T3-receptor complexes (Yaffe, B. M., and Samuels, H. H. (1984) J. Biol. Chem. 259, 6284–6291). In this study we have functionally mapped the elements of the gene which are involved in mediating basal and hormone-regulated expression. Stable transformation studies indicate that transcriptional regulation of the gene by L-T3 is mediated by sequences in the 5'-flanking region. Transient expression studies were performed using a series of chimeric plasmids in which 5'-flanking DNA was ligated to the chloramphenicol acetyltransferase gene. Transient expression occurred only in cells which expressed the endogenous growth hormone gene. Sequences between -104 and +7 were found to be essential for basal expression. One of the most highly conserved regions (-105 to -145) contains elements which further enhance the level of basal expression but are not necessary for regulated expression by L-T3. DNA between -210 and -181 was found to be essential for stimulation by L-T3 and was shown to function most efficiently with the homologous rat growth hormone promoter (-104 to +7). Sequences from -206 to -198 show about 80% homology with a sequence in the 5'-flanking region of two other rat genes which are regulated by thyroid hormone. Glucocorticoid hormones, which also transcriptionally stimulate the rat growth hormone gene, elicted only minimal effects in both stable and transient expression studies. This suggests that the elements which mediate glucocorticoid regulation of the endogenous gene are found either upstream of the cloned 5'-flanking region (1800 base pairs) or 3' of the cap site.

The rat growth hormone gene is one of several genes regulated by the thyroid hormones that have been cloned and sequenced (1–5). Thyroid hormone stimulates growth hormone gene expression in the rat anterior pituitary in vivo (6) and in several clonal strains of growth hormone-producing rat pituitary cells (e.g., GH4, GH3, and GC) (7–9). These cells lines have proven to be highly effective model systems to analyze the biology and structure of the thyroid hormone nuclear receptor which is a DNA binding protein (10–13). In GC cells, 3,5,3'-triiodo-L-thyronine (L-T3) rapidly stimulates the rate of growth hormone gene transcription about 10-fold (14–16) which parallels the binding of L-T3 with its nuclear receptor (16).

In GH, and GC cells (8, 15–19) stimulation of growth hormone gene expression by glucocorticoids is highly dependent on the action of thyroid hormone. In the absence of L-T3, incubation with glucocorticoid hormones (e.g., dexamethasone) for 1–4 h either had no effect or slightly inhibited the transcription rate (16). In contrast, L-T3 + glucocorticoid rapidly and synergistically stimulated growth hormone gene transcription rates which were about 3–4-fold greater than observed with L-T3 alone (15, 16). Under somewhat different culture conditions, others have reported that glucocorticoids can independently stimulate a 4–5-fold increase in the transcription rate in GC cells (14) or mRNA levels in GH3 cells (7). Therefore, both thyroid hormone-dependent and independent effects of glucocorticoid hormone on growth hormone gene expression have been observed.

Using a chimeric gene (pGH-xgpt), we reported that 5'-flanking DNA of the rat growth hormone gene can mediate regulated expression by L-T3 (20). In pGH-xgpt about 1800 bp of 5'-flanking DNA of the rat growth hormone gene were ligated to Escherichia coli DNA which codes for the enzyme, xanthine-guanine phosphoribosyltransferase (XGP-transferase) (21). Stable transformants of GC cells transfected with pGH-xgpt showed L-T3 stimulation of XGP-transferase enzyme and XGP-transferase mRNA levels (20). Since expression in stable transformants may be influenced by the genomic site of integration, chimeric plasmids transiently expressing the chloramphenicol acetyltransferase (CAT) gene (22) were used to localize the regulatory elements in the 5'-flanking region. L-T3 stimulated an increase in CAT activity of about 10–15-fold, and DNA between -236 and -146 bp was found to be essential for regulated expression by thyroid hormone. Deletion studies indicated that these sequences appear to be localized between nucleotides -210 and -181, and these elements were found to function most efficiently with the rat growth hormone promoter. Unlike regulated expression by

1 The abbreviations and trivial names used are: L-T3, 3,5,3'-triiodo-L-thyronine; CAT, chloramphenicol acetyltransferase; dexamethasone, 16a-methyl-9a-fluoro-11b,17,21-trihydroxy-1,4-pregnadiene-3,20-dione; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; neo, neomycin resistance gene; PBS, Dulbecco’s phosphate-buffered saline containing 100 mg/dl glucose; XGP-transferase, xanthine-guanine phosphoribosyltransferase; bp, base pair(s); ACTH, corticotropin; kb, kilobase pair(s).

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Regulated Gene Expression by Thyroid Hormone

thyroid hormone, dexamethasone stimulated only a small increase (1.6-fold) in CAT activity, and no synergistic stimulation with L-T₄ was found. This suggests that the elements which mediate the full effect of glucocorticoid hormones on the endogenous growth hormone gene can be localized either further upstream of -1800 bp or 3' of the cap site.

**EXPERIMENTAL PROCEDURES**

Materials—[14C]Chloramphenicol (45-55 mCi/ml), [α-32P]UTP (600-800 Ci/mmol), and [α-32P]dCTP (600-800 Ci/mmol) were obtained from New England Nuclear. All linkers and enzymes involved in recombinant DNA procedures were obtained from either New England Biolabs or Boehringer Mannheim. Unless indicated otherwise, procedures involving these enzymes used conditions recommended by the suppliers. Acetyl-CoA and α-nitrophenyl β-d-galactopyranoside were obtained from Sigma, chlorophenol red-p-D-galactopyranoside was from Boehringer Mannheim, while all other reagents were of the highest purity available and were obtained from Sigma, Behring Diagnostics, Bio-Rad, Eastman, Fisher, or Boehringer Mannheim.

**Plasmids**—The construction of pGH-xgpt was previously described (20). Vectors expressing CAT under control of the Rous sarcoma virus promoter (pRSV-cat) (22) or the SV40 viral early promoter with thyroid hormone, dexamethasone stimulated only a small increase (1.6-fold) in CAT activity, and no synergistic stimulation with L-T₄ was found. This suggests that the elements which mediate the full effect of glucocorticoid hormones on the endogenous growth hormone gene can be localized either further upstream of -1800 bp or 3' of the cap site.

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Page et al. (4) reported CTTGG in the same position in the growth hormone gene of a different rat strain which is part of the restriction sequence for BstXI. (27) CTTGG was found to cleave rat growth hormone DNA cloned by Barta et al. (1) at -209 indicating a sequence of TGG and not TGA. This was confirmed by chemical sequencing (24). A pRSV-β-gal was cloned using BstXI.

ApCH110 (25) was obtained from Frank Lee of the Dynex Research Institute, Palo Alto, CA. In ApCH110 the lacZ gene was cloned downstream of the SV40 viral enhancer and promoter and has been used in co-transfections with plasmids expressing CAT to correct for variations in transfection efficiency (26). Since pRSV-β-gal functions efficiently in GH cells (27), we also generated a pRSV-β-gal plasmid to monitor the efficiency of transfection in GC cells. pRSV-β-gal was constructed by first excising DNA containing the lacZ gene from ApCH110 and the CAT gene from pRSV-cat by HindIII-BamHI. The HindIII-BamHI fragment containing the lacZ gene was then directionally ligated downstream of the Rous sarcoma virus promoter to form pRSV-β-gal. A pGH-β-gal plasmid was constructed using BstXI.

The growth hormone gene as shown in Fig. 1 in the construction of pGH-cat (1800).

Transient Gene Expression Facilitated by Electroporation—An electroporation chamber was constructed and employed as described by Potter et al. (28) using a plastic spectrophotometer cuvette except that platinum foil and the buffer conditions described below were used. Cells were then harvested at 24-48 h with Dulbecco's modified Eagle's medium containing 10 mM Hepes, pH 7.5, 0.11 mM pyruvate (DHAP), and 10% (v/v) fetal calf serum (20). The cells then received DHAP medium supplemented to 10% (v/v) with thyroid- and steroid hormone-depleted calf serum (20) and were cultured for an additional 24-48 h. Cells were then harvested with trypsin-EDTA (16), washed three times with DHAP medium containing hormone-depleted serum, and the cell pellet (4-6 x 10^6 cells depending on the experiment) was placed on ice. Supercoiled pGH-cat constructs (10-15 µg) was added to 0.4 ml of Dulbecco's phosphate-buffered saline to which 0.1 volume of a 10 g/liter glucose solution had been added (PBS). In several experiments 1.5 µg of pRSV-β-gal or 3 µg of pGH-β-gal was mixed with 15 µg of pGH-cat plasmid in the PBS solution.

The cells were resuspended in sterile chilled PBS containing plasmid, and the mixture was transferred to the electroporation chamber which was maintained at 0 °C. One min later the cells received a pulse of 1.7-2.0 kV at 0.9 mA maximum amperage using an Iso model 494 power supply as described (28). Seven min later the cells were transferred from the cuvette to 1 ml of PBS containing 10% (v/v) hormone-deficient calf serum. After incubating at 25 °C for 7 min, the cells were added to DHAP medium containing hormone-deficient serum and were distributed into 25 cm^2 flasks (Falcon). In general, about 10^6 cells for each flask to be inoculated were used for electroporation. The most efficient transfection occurred when 20-30% of the cells were stained by trypan blue. When serial electroporations were carried out with the same pooled cell population, the increase in the levels of XGP-transferase enzyme and XGP-transferase gene expression is secondary to transcriptional control and not due to an effect on XGP-transferase mRNA stabilization. pGH-xgpt-transfected GC cells were incubated ±5 nM L-T_3 for 1 h, and the nuclei were then incubated in vitro with [α-³²P]UTP as described (16). The labeled RNA was then hybridized to filter-immobilized rat growth hormone cDNA or DNA containing the E. coli XGP-transferase gene. Stimulation of transcription of the XGP-transferase gene expression is secondary to transcripational control and not due to an effect on XGP-transferase mRNA stabilization. pGH-xgpt-transfected GC cells were incubated ±5 nM L-T_3 for 1 h while the remaining flasks served as controls. Nuclei were isolated, incubated with [α-³²P]UTP at 29 °C, and the [³²P]chloramphenicol was quantitated in a liquid scintillation counter and the results expressed as percent acetylated forms of chloramphenicol and, therefore, the cell extracts were not heated to prevent deacetylation. Heating cell extracts to 55-60 °C generally had no effect on CAT activity or in some cases the activity was reduced by 15-20%.

CAT reactions were performed with ethyl acetate and chromatographed on silica gel thin layer plates using chloroform:methanol (95:5) (22). After autoradiography the acetylated and nonacetylated [³²P]chloramphenicol was quantitated in a liquid scintillation counter and the results expressed as percent acetylated forms of chloramphenicol. β-Galactosidase was assayed (30) with 50-75 µg of lysozyme protein using either o-nitrophenol-β-D-galactopyranoside or chlorophenol red β-D-galactopyranoside. Histochemical staining for β-galactosidase was used to assess the percentage of cells involved in transfection after electroporation. This was performed essentially as described by Lund-Hansen et al. (31) except that the polyvinyl alcohol used was grade G08/140 (Wacker Chemicals U. S. A., Inc.) and the pH of the McIlvaine citrate-phosphate buffer was 7.0. Cell monolayers were incubated at 37 °C in a humidified atmosphere for 18 h with 5-hromo-4-chloro-3-indolyl-β-D-galactopyranoside and then photographed.

RESULTS

Thyroid Hormone Stimulates XGP-transferase Gene Transcription in GC Cells after Stable Transformation with pGH-xgpt—pGH-xgpt was used to transfect GC cells, and stable transformants were isolated using mycophenolic acid and xanthine (20). In these transformants L-T_3 stimulated an increase in the levels of XGP-transferase enzyme and XGP-transferase mRNA which was correctly initiated at the cap site of the growth hormone gene in the chimeric plasmid (20). The in vitro transcription study shown in Fig. 3 indicates that the increase in XGP-transferase gene expression is secondary to transcriptional control and not due to an effect on XGP-transferase mRNA stabilization. pGH-xgpt-transfected GC cells were incubated ±5 nM L-T_3 for 1 h, and the nuclei were then incubated in vitro with [α-³²P]UTP as described (16). The labeled RNA was then hybridized to filter-immobilized rat growth hormone cDNA or DNA containing the E. coli XGP-transferase gene. Stimulation of transcription of the

Graphical representation of the figure.

Fig. 3. In vitro transcription of the XGP-transferase and growth hormone (GH) genes. Stable transformants of GC cells transfected with pGH-xgpt were cultured for 1 h in 35 cm^2 flasks with DHAP medium supplemented to 10% (v/v) with hormone-depleted calf serum. Half of the flasks from each group were incubated with 5 nM L-T_3 for 1 h while the remaining flasks served as controls. Nuclei were isolated, incubated with [α-³²P]UTP at 29 °C, and the [³²P]chloramphenicol was quantitated in a liquid scintillation counter and the results expressed as percent acetylated forms of chloramphenicol and, therefore, the cell extracts were not heated to prevent deacetylation. Heating cell extracts to 55-60 °C generally had no effect on CAT activity or in some cases the activity was reduced by 15-20%.

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Other Procedures—Quantitation of mRNA levels and estimation of in vitro transcription using isolated nuclei were carried out as previously described (16) and are detailed in the appropriate figure legends.

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The labeled RNA was then hybridized to filter-immobilized rat growth hormone cDNA or DNA containing the E. coli XGP-transferase gene. Stimulation of transcription of the
chimeric pGH-xgpt gene (L-T3, 21.7 ppm; control, 2.5 ppm) paralleled that of the endogenous growth hormone gene (L-T3, 32.8 ppm; control, 3.5 ppm). This further supports the notion that transcriptional control can fully account for L-T3 stimulation of growth hormone gene expression (16, 20) which is mediated by sequences present in the 1800-bp 5′-flanking region of the gene.

Electroporation-mediated Transient Gene Expression in GC Cells—To localize the elements which mediate basal and hormonal stimulation of the rat growth hormone gene, pGH-xgpt plasmids were constructed containing 5′-flanking extensions to −530, −312, and −237. These plasmids were used to generate stable transformants of GC cells as previously described (20). Although transformed colonies were pooled for study, the magnitude of stimulation of XGP-transferase by L-T3 varied among different transfections with the same plasmid. This might have resulted from differences in the genomic site(s) of integration of the plasmid. Alternatively, even though many colonies were pooled, they might have been derived from only a few clones since GC cells tend to detach and reseed the plate to form new colonies. Because of these problems, we explored the use of transient gene expression to map the elements in the 5′-flanking region which mediate basal and hormone-regulated expression. The construction of plasmids containing various 5′-flanking extensions of the rat growth hormone gene ligated to the CAT gene is outlined in Figs. 1 and 2 and is detailed under "Experimental Procedures."

pRSV-βgal and pRSV-cat were used to monitor various transfection procedures, and electroporation (28) was found to be more efficient in GC cells than DEAE-dextran (27) or calcium phosphate precipitation (20, 32). Fig. 4 illustrates a cytochemical analysis of β-galactosidase expression in GC cells 48 h after electroporation with 10 μg of pRSV-βgal. Approximately 10% of the cells expressed β-galactosidase (Fig. 4) while control cells exposed to the procedure without plasmid showed no histochemical evidence for enzyme activity (not illustrated). From a variety of electroporation studies we estimate that between 5 and 15% of cells exhibit transient expression. Although these and the other plasmids used in this study do not replicate in GC cells, the cells continue to divide over the 24–72-h incubation period after electroporation. This includes cells which exhibit transient expression (see upper right of Fig. 4) as well as those which show no enzyme activity. Since L-T3 stimulates GC cell replication, the effect of cell growth needs to be taken into consideration when analyzing the effects of thyroid hormone on transient gene expression. If L-T3 had no effect on transient expression of a plasmid, the amount of enzyme activity/μg of cell protein would be expected to be reduced by thyroid hormone if identical amounts of cell protein are assayed. This would occur since L-T3 stimulates the cells to replicate which decreases the relative amounts of plasmid and gene product per cell or per μg of protein assayed. Similarly, if L-T3 stimulated transient expression of a plasmid, the increase in cell division would result in an apparent response which is lower than the actual level of hormonal stimulation.

This "dilutional" effect of cell replication on the response is illustrated by the effect of L-T3 on CAT expression in cells transfected with pRSV-cat and pA10G-cat (Fig. 5). GC cells, transfected with either pRSV-cat or pA10G-cat, were incubated with L-T3 for 48 h. In each case L-T3 appears to reduce the extent of CAT expression when identical amounts of cell protein were assayed for CAT activity (40 μg). However, Table I shows that when the extent of CAT activity is corrected for the effect of cell replication, L-T3 had no effect on the level of expression of the CAT gene in cells transfected with pRSV-cat or pA10G-cat. In contrast with pRSV-cat and pA10G-cat, cells transfected with pGH-cat(−1800) showed L-T3 stimulation of CAT activity (Fig. 5). Because of the dilutional effect of cell replication, stimulation of CAT activity by L-T3 in cells transfected with pGH-cat(−1800) likely underestimates the extent of regulation by hormone (Table I). Both uncorrected and replication corrected results are shown for each of the remaining studies to be presented.

Influence of L-T3 and Dexamethasone on Transient Gene Expression of pGH-cat Plasmids—Transfection using 10–15 μg of pGH-cat(−1800) was found to give the highest hormonal stimulation of CAT relative to basal activity. Higher amounts of plasmid (greater than 25 μg) resulted in a partial nonlinear increase in basal CAT activity and a reduction in the extent

![Fig. 4. Histochemical staining for β-galactosidase activity in cells transfected with pRSV-βgal. GC cells (30 × 10⁶) were transfected with pRSV-βgal (10 μg) by electroporation. The cells were cultured for 48 h in DHAP medium containing L-T3 and then stained for β-galactosidase using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Cells expressing large amounts of β-galactosidase are darkly stained in the figure. GC cells transfected with pRSV-cat showed no staining for β-galactosidase activity (not shown).](image-url)

![Fig. 5. Effect of L-T3 on CAT expression in GC cells transfected with pRSV-cat, pA10G-cat, and pGH-cat(−1800). GC cells were cultured with hormone-depleted medium for 24 h and then transfected with 5 μg of pRSV-cat, 10 μg of pA10G-cat, or 10 μg of pGH-cat(−1800). Thirty million cells were used for each electroporation (2000 V), and the trypan blue uptake was between 25 and 30%. Cells from each transfection were divided into 6 flasks, and half of each group was incubated with 5 nM L-T3 for 48 h. Cell lysates containing 40 μg of protein from each flask were assayed for CAT activity by incubating the samples for 12 h at 37 °C with 10 nm acetyl-CoA and 0.15 μCi of [14C]chloramphenicol. Representative thin layer plates showing the extent of [14C]chloramphenicol acetylation for each of the transfected cell groups are shown in the figure. The average percent acetylation of [14C]chloramphenicol for each group (both uncorrected and corrected for the effect of L-T3 on cell replication) is shown in Table I.](image-url)
Transfected cells was: pRSV-cat (control, 185; L-T\textsubscript{3}, 455); pA10-cat2 (control, 152; L-T\textsubscript{3}, 425). Forty \( \mu \)g of lysate protein was assayed for CAT activity, and the uncorrected results represent the mean of the replicate flasks which showed less than 5% variation. The corrected activity was adjusted for the effect of L-T\textsubscript{3} on cell replication by multiplying the uncorrected percent acetylation by the ratio of the amount of cell lysate protein recovered from the hormone-incubated flasks/the protein recovered from the cells incubated without hormone. The -fold stimulation of corrected activity was adjusted from the cells incubated without hormone. The -fold stimulation of recovered CAT activity was taken as the ratio of CAT activity of the hormone/control cells.

\[
\begin{array}{llllll}
\text{Plasmid} & \text{Acetylation} & \text{Stimulation} \\
& \text{Uncorrected} & \text{Corrected} & \text{Uncorrected} & \text{Corrected} & -fold \\
pRSV-cat & & & & & \\
\text{Control} & 13.6 & 13.6 & & & \\
L-T\textsubscript{3} & 9.2 & 12.9 & 0.38 & 0.95 & \\
pA10-cat2 & 8.3 & 8.3 & & & \\
L-T\textsubscript{3} & 3.9 & 8.0 & 0.48 & 0.96 & \\
pGH-cat(-1800) & 1.5 & 1.5 & & & \\
\text{Control} & 10.6 & 29.6 & 7.1 & 19.7 & \\
L-T\textsubscript{3} & & & & & \\
\end{array}
\]

\section*{TABLE II}
Effect of L-T\textsubscript{3} on enzyme expression in GC cells transfected with pGH-cat(-1800) and pGH-\beta-gal(-1800)

GC cells were transfected with 10 \( \mu \)g of pGH-cat(-1800) or with 10 \( \mu \)g of pGH-cat(-1800) + 14 \( \mu \)g of pGH-\beta-gal(-1800). Triplicate flasks from each transfection were cultured with 5 nM L-T\textsubscript{3} for 48 h while cells which received no hormone served as controls. Fifty \( \mu \)g of lysate protein was assayed for CAT activity (c) or \( \beta \)-galactosidase (9) using \( o \)-nitrophenyl-\( \beta \)-D-galactopyranoside as an internal standard in co-transfection experiments (33). These observations precluded the use of pRSV-\beta-gal or \Delta CH110 as internal markers to correct for possible variations among experiments with different pGH-cat plasmids carried out at different times. Therefore, when comparing the extent of CAT expression of different 5'-flanking deletions, transfections were carried out with the plasmids of interest in the same experiment. In addition, the transfection experiments reported have been carried out at least 4 times with similar relative differences in the extent of basal and hormone-regulated expression.

\[
\begin{array}{llllll}
\text{Plasmid} & \text{Enzyme activity} & \text{Stimulation} \\
& \text{Uncorrected} & \text{Corrected} & \text{Uncorrected} & \text{Corrected} & -fold \\
pGH-cat(-1800) & & & & & \\
\text{Control (c)} & 1.6 & 1.6 & & & \\
L-T\textsubscript{3} (c) & 7.7 & 17.5 & 4.8 & 10.9 & \\
pGH-\beta-gal(-1800) & & & & & \\
\text{Control (c)} & 0.81 & 0.81 & & & \\
L-T\textsubscript{3} (c) & 4.1 & 8.8 & 5.1 & 10.9 & \\
pGH-\beta-gal(-1800) & & & & & \\
\text{Control (g)} & 0.12 & 0.12 & & & \\
L-T\textsubscript{3} (g) & 0.52 & 1.1 & 4.3 & 9.2 & \\
\end{array}
\]

Effects using higher concentrations of dexamethasone (100-200 nM) gave similar results. The effect of L-T\textsubscript{3} + dexamethasone on CAT activity was additive and not synergistic as reported for the endogenous growth hormone gene (8, 15-18). The absence of a large dexamethasone effect on stimulating CAT activity is not an inherent property of transient gene expression since no significant effect of dexamethasone on XGP-transferase mRNA levels was observed in pGH-XGPT-transformed cells (Fig. 6).
TABLE III

| Incubation | Acetylation | Stimulation |
|------------|-------------|-------------|
|            | Uncorrected | Corrected   | Uncorrected | Corrected   |
| 24 h       |             |             |             |             |
| Control    | 1.6         | 1.6         |             |             |
| L-T3       | 8.1         | 12.1        | 5.1         | 7.6         |
| DEX        | 2.5         | 2.5         | 1.6         | 1.6         |
| L-T3 + DEX | 14.9        | 6.2         | 9.3         |             |
| 48 h       |             |             |             |             |
| Control    | 2.4         | 2.4         |             |             |
| L-T3       | 16.4        | 31.3        | 6.8         | 13.0        |
| DEX        | 4.1         | 4.1         | 1.7         | 1.7         |
| L-T3 + DEX | 18.4        | 34.7        | 7.7         | 14.5        |
| 72 h       |             |             |             |             |
| Control    | 2.1         | 2.1         |             |             |
| L-T3       | 17.9        | 33.1        | 8.5         | 15.8        |
| DEX        | 3.6         | 3.6         | 1.7         | 1.7         |
| L-T3 + DEX | 19.9        | 36.6        | 9.5         | 17.4        |

Fig. 6. XGP-transferase mRNA levels in transfected cells incubated with L-T3, dexamethasone (DEX), and L-T3 + dexamethasone. Duplicate flasks of pooled colonies of GC cells, transfected with pGH-xgp, were incubated with either 5 nM L-T3, 50 nM dexamethasone, or L-T3 + dexamethasone for 48 h. Cells which received no hormone served as controls. Cytoplasmic RNA was isolated, dotted to nitrocellulose (51), and hybridized to nick-translated pSV2-gpt (16, 20). The nitrocellulose was autoradiographed for 12 h at −80°C using Kodak X-Omat AR film and a Lightning Plus intensifying screen.

Fig. 7. Influence of L-T3 and dexamethasone (DEX) on CAT activity in GC cells transfected with pGH-cat plasmids with different 5'-flanking extensions. Forty million GC cells were transfected with identical molar amounts (11.5–15 μg) of pGH-cat plasmids with 5'-flanking DNA extending to −104, −145, −181, −209, −237, and −1800 bp. After electroporation, the cells were cultured in duplicate with 5 nM L-T3, 50 nM dexamethasone, or L-T3 + dexamethasone for 48 h, and 50 μg of cell lysate protein was assayed for CAT activity. The figure shows the extent of [14C]chloramphenicol acetylation in the duplicate flasks from the cells transfected with A, pGH-cat(-237) (autoradiographed for 14 h) and B, pGH-cat(-145) (autoradiographed for 24 h). Quantitation of the results for each plasmid is shown in Table IV.

same time. Basal CAT activity was similar for cells transfected with pGH-cat(-237) and pGH-cat(-145) while the level with pGH-cat(-104) was reduced about 65%. In five independent experiments basal expression of cells transfected with pGH-cat(-104) was 25–40% of that found with pGH-cat(-145). The lower levels of basal expression with pGH-cat(-104) were at least 5–10-fold greater than that seen pSV0-cat. pSV0-cat lacks the SV40 viral enhancer and promoter (22) and showed essentially no CAT expression in GC cells. When corrected for effects of L-T3 on cell growth, thyroid hormone was found to have no effect on CAT expression in cells transfected with pGH-cat(-104) or pGH-cat(-145). L-T3 stimulated CAT activity to similar maximal levels in cells transfected with pGH-cat(-1800), pGH-cat(-237), or pGH-cat(-209). In cells transfected with pGH-cat(-181) the absolute L-T3-stimulated levels of CAT (after subtraction of basal activity) were reduced about 50%, and the fold stimulation by 65%, while the basal enzyme activity remained unchanged. This suggests that sequences responsible for L-T3 stimulation of CAT are localized between −210 and −146 and that DNA between −210 and −181 has an important effect in mediating regulated expression by thyroid hormone.

In contrast with L-T3, dexamethasone was found to have both inhibitory as well as weak stimulatory effects (Table IV). Dexamethasone had significant inhibitory effect on CAT expression in cells transfected with pGH-cat(-104). The extent of CAT inhibition by dexamethasone was somewhat less in cells transfected with pGH-cat(-145) and did not occur or
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Effect of hormone on CAT expression in GC cells transfected with pGH-cat plasmids containing different 5'-flanking DNA extensions

GC cells (40 x 10⁶) were transfected with 11.5-15 µg of the plasmids indicated so as to maintain identical molar amounts in each transfection. Cells from each transfection were cultured with 5 nM L-T₃, 50 nM dexamethasone (DEX), or L-T₃ + dexamethasone, while cells which received no additions served as controls. After 48 h of incubation, the cells were harvested and assayed for CAT activity using 50 µg of lysate protein. Fig. 7 shows the results for pGH-cat(-237) and pGH-cat(-145). The amount of lysate protein recovered from each group was similar to that reported for the 48-h point in Table III, and the effect of L-T₃ on cell replication is reflected by the ratio of the corrected/uncorrected percent acetylation. The amount of protein/flask in cells cultured with 50 µM dexamethasone was essentially identical to the control cells. The results reflect the average of duplicate flasks, each of which varied less than 7%.

| Plasmid                  | Acetylation | Stimulation |
|--------------------------|-------------|-------------|
|                           | Uncorrected | Corrected   | %          | -fold     |
| pGH-cat(-104)            |             |             |           |          |
| Control                  | 0.64        | 0.34        |            |           |
| L-T₃                     | 0.22        | 0.53        | 0.41      | 0.98      |
| DEX                      | 0.25        | 0.25        | 0.46      | 0.46      |
| L-T₃ + DEX               | 0.13        | 0.29        | 0.24      | 0.54      |
| pGH-cat(-145)            |             |             |           |          |
| Control                  | 1.5         | 1.5         |            |           |
| L-T₃                     | 0.66        | 1.6         | 0.44      | 1.1       |
| DEX                      | 0.98        | 0.98        | 0.35      | 0.65      |
| L-T₃ + DEX               | 0.39        | 0.95        | 0.26      | 0.63      |
| pGH-cat(-1800)           |             |             |           |          |
| Control                  | 1.6         | 1.6         |            |           |
| L-T₃                     | 2.8         | 5.3         | 1.7       | 3.3       |
| DEX                      | 1.5         | 1.5         | 0.81      | 0.81      |
| L-T₃ + DEX               | 3.0         | 6.5         | 1.9       | 4.1       |
| pGH-cat(-209)            |             |             |           |          |
| Control                  | 1.4         | 1.4         |            |           |
| L-T₃                     | 7.9         | 16.4        | 5.6       | 11.7      |
| DEX                      | 1.5         | 1.5         | 1.1       | 1.1       |
| L-T₃ + DEX               | 8.5         | 17.4        | 6.1       | 12.4      |
| pGH-cat(-237)            |             |             |           |          |
| Control                  | 1.7         | 1.7         |            |           |
| L-T₃                     | 7.2         | 17.7        | 4.2       | 10.4      |
| DEX                      | 2.8         | 2.8         | 1.6       | 1.6       |
| L-T₃ + DEX               | 8.2         | 20.6        | 4.8       | 12.1      |
| pGH-cat(-1800)           |             |             |           |          |
| Control                  | 1.4         | 1.4         |            |           |
| L-T₃                     | 8.5         | 16.3        | 6.1       | 11.6      |
| DEX                      | 2.4         | 2.4         | 1.7       | 1.7       |
| L-T₃ + DEX               | 3.2         | 17.6        | 6.6       | 12.6      |

Effect of 3' deletions in the 5'-flanking region on the regulated expression by CAT by L-T₃

GC cells were transfected with each of the plasmids indicated in the table which are described in the text. pGH-cat(-237) was used at 15 µg, and the micrograms of DNA of the other plasmids were adjusted to maintain identical molar amounts in each transfection. Electroporation was performed with 50 x 10⁶ GC cells which were then distributed into six flasks and incubated in triplicate 5 nM L-T₃. After 48 h, the cells were harvested, and 50 µg of lysate protein was assayed for CAT activity. The transfection results reflect the mean of triplicate flasks, each of which varied less than 7%. The effect of L-T₃ on cell replication is reflected by the ratio of the percent acetylation in the corrected/uncorrected amounts given in the table.

| Plasmid                  | Acetylation | Stimulation |
|--------------------------|-------------|-------------|
|                           | Uncorrected | Corrected   | %          | -fold     |
| pGH-cat(-104)            |             |             |           |          |
| Control                  | 0.44        | 0.44        |            |           |
| L-T₃                     | 0.23        | 0.48        | 0.52      | 1.1       |
| pGH-cat(-104; -237/-530) |             |             |           |          |
| Control                  | 0.41        | 0.41        |            |           |
| L-T₃                     | 0.20        | 0.43        | 0.49      | 1.0       |
| pGH-cat(-104; -130/-236) |             |             |           |          |
| Control                  | 0.39        | 0.39        |            |           |
| L-T₃                     | 1.5         | 2.9         | 3.9       | 7.4       |
| pGH-cat(-104; -130/-236) |             |             |           |          |
| Control                  | 0.81        | 0.81        |            |           |
| L-T₃                     | 2.8         | 5.3         | 3.5       | 6.6       |
| pGH-cat(-237)            |             |             |           |          |
| Control                  | 1.3         | 1.3         |            |           |
| L-T₃                     | 4.4         | 9.3         | 3.4       | 7.2       |
| pA10o-cat₁               |             |             |           |          |
| Control                  | 3.3         | 3.3         |            |           |
| L-T₃                     | 1.7         | 3.1         | 0.52      | 0.94      |
| pA10o-cat₁(-237/-530)    |             |             |           |          |
| Control                  | 4.2         | 4.2         |            |           |
| L-T₃                     | 2.5         | 4.1         | 0.60      | 0.98      |
| pA10o-cat₁(-146/-236)    |             |             |           |          |
| Control                  | 3.0         | 3.0         |            |           |
| L-T₃                     | 1.9         | 3.1         | 0.63      | 1.0       |
-236 bp and that sequences from -105 to -145 and -237 to -530 do not play an important role in mediating regulated expression by thyroid hormone. DNA from -146 to -236 and -237 to -530 was also ligated into the BglII site just upstream of the enhancerless SV40 promoter in pA\textsubscript{0}-cat\textsubscript{2} (23, 35) to form pA\textsubscript{0}-cat\textsubscript{2}(-146/-236) and pA\textsubscript{0}-cat\textsubscript{2}(-237/-530). No regulated expression by thyroid hormone was observed with either of the pA\textsubscript{0}-cat\textsubscript{2} recombinants (Table V). This suggests that DNA from -146 to -236, which mediates regulated expression by thyroid hormone, functions more efficiently with or requires the homologous promoter sequences found in pGH-cat\textsubscript{2} (-104). No stimulation of CAT by dexamethasone ± L-T\textsubscript{3} was found with any of the pA\textsubscript{0}-cat\textsubscript{2} plasmids (not illustrated).

**Cell-specific Factors Influence Regulatory Elements in the 5'-Flanking Region of the Rat Growth Hormone Gene—** A number of other rat pituitary cell lines, which express the growth hormone gene, were transfected with pGH-cat(-1800) and examined for basal and hormonal regulation of CAT activity. The clonal strains examined were GH\textsubscript{1} (9), GH\textsubscript{C\textsubscript{1}} (36), and GH\textsubscript{4} (7). The basal expression of CAT in GH\textsubscript{C\textsubscript{1}} cells was slightly lower (about 50\%) than that found in GC cells while GH\textsubscript{1} and GH\textsubscript{4} cells were similar. However, each of these cell lines showed the same pattern of regulated expression of CAT activity by L-T\textsubscript{3} and/or dexamethasone as GC cells (not shown). Rat2 fibroblasts (37), rat hepatoma cells (38), and ACTH-producing A1T20 mouse pituitary cells (39) all showed high levels of CAT expression when transfected with pRSV-cat. However, no basal or hormone-regulated levels of CAT enzyme were detected when these nongrowth hormone-producing cells were transfected with pGH-cat(-1800). This suggests that cell-specific trans-acting factors interact with 5'-flanking DNA of the rat growth hormone gene to regulate cell-specific expression of the gene.

**DISCUSSION**

In this study we have functionally mapped the 5'-flanking regions of the rat growth hormone gene which are important in mediating basal and regulated expression by hormone. Using GC cells transfected with pGH-xgpt, we showed that regulated expression by thyroid hormone was mediated by sequences found in the cloned 1800-bp 5'-flanking region (29). Subsequently, using GC cells transfected with pGH-neo plasmids, Crew and Spindler (40) reported that thyroid hormone stimulation could occur with only 235 bp of 5'-flanking DNA of the rat growth hormone gene. However, the basal level of expression and the extent of stimulation by thyroid hormone was low compared with GC cells transfected with pGH-neo plasmids containing longer 5'-flanking extensions (40). In attempting to map the functional elements in the 5'-flanking region, we found that pooled colonies of stable transformants generated by the same plasmid showed varying amounts of basal and hormone-regulated expression. Therefore, we could not accurately map these elements by generating stable transformants with pGH-xgpt plasmids containing different 5'-flanking deletions. In contrast, transient expression of pGH-cat plasmids was found to give consistent relative differences in CAT expression, and we thus used this approach to map the functional elements in the 5'-flanking region.

Sequences between -145 and +7 were found to play a dominant role in determining the level of basal expression since basal CAT levels with pGH-cat(-145) were similar to CAT plasmids containing longer 5'-flanking extensions. However, CAT expression with pGH-cat(-104) was about 3-fold less than with pGH-cat(-145), suggesting that DNA between -145 and -104 contain elements which enhance the level of basal expression. Although these sequences are not necessary for regulated expression by thyroid hormone (Table V), DNA from -145 to -105 is one of the most highly conserved regions of the rat, human, and bovine growth hormone genes (1, 4, 5, 34). In a recent study (41) we examined the binding of rat growth hormone 5'-flanking DNA with nuclear proteins from GC, GH\textsubscript{1}, and GH\textsubscript{C\textsubscript{1}} cells and rat cells which do not express the growth hormone gene (H4 hepatoma cells and Rat2 fibroblasts). Only nuclear proteins from the growth hormone-producing cells formed a sequence-specific protein-DNA complex with a fragment extending from -145 to +7. This cell-specific protein(s) generated a DNase I-resistant footprint between -95 and -68 which spans the "CAAT" homology. This suggests that the first 104 bp of 5'-flanking DNA contain elements which may be important for cell-specific expression of the gene. Whether the highly conserved sequences between -145 and -105 influence cell-specific expression, which is reflected in GC cells as an increase in basal activity, or whether they mediate effects by other factors is under investigation.

Our stable and transient expression studies imply that the elements which mediate the full effect of glucocorticoid hormones on the endogenous growth hormone gene are not localized in the cloned 5'-flanking region. First, glucocorticoid stimulation of XGP-transferase mRNA in GC cells transfected with pGH-xgpt was not observed even when L-T\textsubscript{3} was present (Fig. 6). Similarly, Crew and Spindler (40) found that dexamethasone either had no effect or slightly inhibited the level of neo transcripts in GC cells transfected with pGH-neo plasmids. In transient expression studies with pGH-cat(-1800) or pGH-cat(-237) (Tables III and IV), dexamethasone stimulated an increase in CAT expression which was generally about 1.6-fold, and L-T\textsubscript{3} + dexamethasone did not synergistically stimulate CAT expression. The 1.6-fold stimulation by dexamethasone is much lower than in other studies where glucocorticoid hormones have been reported to stimulate expression of the endogenous growth hormone gene 5-20-fold without added L-T\textsubscript{3} (7, 14). The small effect of dexamethasone on CAT expression is similar to the response reported by Robins et al. (42) in Ltk- cells transfected with a chimeric gene containing 496 bp of 5'-flanking DNA of the human growth hormone gene ligated to the herpes simplex virus tk gene.

5'-Flanking DNA of the rat growth hormone gene contains the sequence TGTCTCT in an inverted orientation in the noncoding strand from -260 to -265 which is part of a consensus sequence of a number of glucocorticoid regulatory elements (43). However, pGH-cat(-237) showed the same dexamethasone response as pGH-cat(-1800) (Table IV) indicating that the sequence containing TGTCTCT on the noncoding strand does not mediate regulated expression of CAT in these chimeric plasmids. Interestingly, dexamethasone inhibited expression of pGH-cat(-104) and pGH-cat(-145) indicating the presence of both positive and negative regulatory elements in the first 237 bp of 5'-flanking DNA (Table IV). These negative elements may explain the observations that, in the absence of L-T\textsubscript{3}, dexamethasone may inhibit growth hormone gene transcription (16) or the expression of neo transcripts in cells transfected with pGH-neo (40). Differential regulation of these positive and negative elements by components of the medium may account for differences in the extent of glucocorticoid stimulation of growth hormone gene expression reported in various studies (7, 8, 14-19).

Although dexamethasone weakly stimulated CAT expression, no synergistic stimulation of the integrated XGP-trans-
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ferase gene or the transiently expressed CAT gene was found when cells were cultured with L-T₃ + glucocorticoid (Fig. 6, Tables III and IV). This suggests that a glucocorticoid regulatory element(s) is located either further upstream of −1800 bp or 3' of the rat growth hormone gene cap site as suggested by studies in Ltk⁻ cells using a rat growth hormone gene devoid of 5'-flanking sequences (44). Glucocorticoid receptor binds poorly to the 5'-flanking region of the human growth hormone gene while a high affinity site has been identified in the first intron of the human gene (43, 45). This region contains the sequence TGTCCCT in the coding strand and can function as a glucocorticoid regulatory element when positioned upstream of a heterologous promoter (46). TGTCCCT is also found in the coding strand in the first intron of the rat growth hormone gene from +233 to +238 (1, 4). Whether this region contains a functional glucocorticoid response element as has been suggested for sequences in the first intron of the human growth hormone gene is under investigation.

In contrast with glucocorticoids, regulated expression of the endogenous growth hormone gene by thyroid hormone appears to be fully mediated by sequences in the 5'-flanking region. This notion is supported by the observation that L-T₃ stimulates similar increases in the levels of growth hormone mRNA and XGP-transfase mRNA (20) and in the transcription rates of these genes (Fig. 3). Our transient expression studies support the notion that DNA between −236 and −146 is essential for regulated expression by thyroid hormone. These sequences appear to be predominantly located between −210 and −181 since L-T₃ stimulation of CAT in cells transfected with pGH-cat(−181) was about 4-5-fold lower than with pGH-cat(−209) or pGH-cat(−237). Comparison of sequences between −210 and −181 of the rat growth hormone gene with the two other thyroid hormone-responsive rat genes which have been sequenced indicates some homology in the 5'-flanking regions of the growth hormone gene (1, 4) (−200-GC(A/G)AAAGCC−190), the α-myosin heavy chain gene (3) (−100-GAGCCAAGGC−129), and the gene which codes for the hepatic protein "Spot 14" (2) (−110-GACCAAACGC−129). However, it is not known whether 5'-flanking DNA of the α-myosin heavy chain and the Spot 14 genes containing these sequences are involved in regulated expression by L-T₃ since transfection studies with these genes have not yet been reported.

In a recent report Nelson et al. (47) examined the expression of CAT plasmids ligated to 5'-flanking DNA of the rat prolactin and growth hormone genes to identify "enhancer" elements which determine expression of these genes. The amount of CAT expression was maximal in constructs containing at least 235 bp of 5'-flanking growth hormone DNA while deletion to −181 bp markedly lowered the response. These studies using GC, GH, or GH cells were carried out with media supplemented with serum which was not depleted of hormone, and no effect of hormone was examined (47). The amount of thyroid hormone normally present in calf serum or fetal calf serum is sufficient to stimulate growth hormone gene expression to about 85% of the maximal level (9, 18). Therefore, the reported difference in expression of the −235 and −181 growth hormone 5'-flanking CAT plasmids (47) can be accounted for by the sequences which are important for thyroid hormone action which we have shown to reside between −210 and −181 (Table IV).

DNA fragments from −236 to −146 or −236 to −130 each mediate the same degree of regulated expression by thyroid hormone when ligated in the correct orientation upstream of the first 104 bp of 5'-flanking DNA in pGH-cat(−104) (Table V). The initial nucleotide of the two fragments, relative to the cap site, differs by 16 bp which is an odd multiple of the number of base pairs per turn of B-DNA (10.5 bp) (48). Therefore, when ligated to pGH-cat(−104), the sequences involved in regulated expression by thyroid hormone in pGH-cat(−104;−146/236) and pGH-cat(−104;−130/236) will be on different sides of the helix relative to the promoter sequences in pGH-cat(−104). Thus, unlike the SV40 viral early enhancer-promoter (49), our results suggest that stereospecific alignment of "regulatory" and "promoter" sequences on the same side of the helix is not critical for stimulation of gene expression by thyroid hormone.

In contrast with the rat growth hormone promoter, DNA from −236 to −146 did not mediate regulated expression by L-T₃ when ligated upstream of the heterologous SV40 viral promoter in pAIO-cat (Table V). Therefore, sequences between −236 and −146 which influence regulated expression by thyroid hormone appear to function more efficiently with the homologous rat growth hormone promoter. This implies that DNA between −236 and −146 as well as sequences between −104 and the cap site are necessary for "full" regulated expression by L-T₃. Using gel electrophoretic shift assays (41), [³²P]DNA between −236 and −146 was found to form a sequence-specific protein-DNA complex with a low abundant nuclear protein. Unlike the protein(s) which binds between −95 and −68, this protein(s) is not cell type specific, and its abundance in different cell lines paralleled the level of thyroid hormone nuclear receptor. Studies are in progress using site-directed mutagenesis to define the relative importance and possible interrelationship of these regions in mediating full regulated expression by thyroid hormone and the functional significance of the proteins which bind with these sequences.

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