Regulated expression of chimaeric genes containing the 5'-flanking regions of human growth hormone-related genes in transiently transfected rat anterior pituitary tumor cells

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ABSTRACT

The expression and hormonal regulation of chimaeric genes containing the 5'-flanking regions of the normal human growth hormone (hGH-1), the variant hGH (hGH-2) and chorionic somatomammotropin (hCS-1) genes fused to the chloramphenicol acetyl transferase (CAT) gene has been examined after transient transfection into cultured rat pituitary (GC), and non-pituitary (HeLa and Rat 2) tumor cells. As assessed by levels of CAT activity, the hGH-1 and hCS-1 gene hybrids were expressed at 5- to 25-fold higher levels in GC cells than in HeLa or Rat 2 cells. The hGH-2 gene hybrid was expressed at very low levels in all 3 cell types. Triiodothyronine treatment of transiently transfected GC cells had little effect on CAT activity from the hGH-1 gene hybrid but increased CAT activity from the hCS-1 gene hybrid. A slight but significant increase in CAT expression was detected with both genes after dexamethasone treatment. The data indicate that elements present on the hGH-1 and hCS-1 genes' 5'-flanking DNA are required for the efficient expression of these genes in GC cells.

INTRODUCTION

The human growth hormone (hGH) and chorionic somatomammotropin (hCS) genes are highly homologous genes that have evolved from a common ancestor gene (reviewed in ref. 1). The normal hGH gene, hGH-1, is expressed in the pituitary (2), whereas the hCS genes, hCS-1 (3) and hCS-2 (4), and hGH-2, an hGH variant gene (P. Seeburg, personal communication), are expressed in the placenta. The factors that regulate the tissue-specific expression of these genes have not been identified. In addition, the circulating levels of hGH appear to be regulated by a number of factors, including thyroid and glucocorticoid hormones (5-7); however, the mechanisms which control hGH synthesis are unknown. No information is currently available concerning the hormonal regulation of hCS synthesis.

In order to obtain information about the tissue-specific and hormonal regulation of the hGH and hCS genes we have been studying the behavior of these genes after transfer to cultured rat anterior pituitary tumor cells (GC) (8,9). GC cells synthesize and secrete rat growth hormone (rGH) and rGH mRNA

synthesis has been shown to be regulated at the level of transcription by both thyroid and glucocorticoid hormones in these cells (10). We have observed previously that the intact hGH gene containing 496 bp and 628 bp of 5'- and 3'-flanking DNA and structural gene sequences is expressed in GC cells after stable integration (9). The hGH gene transcript is initiated correctly and hGH mRNA levels are increased by dexamethasone (Dex) and decreased by triiodothyronine (T₃) treatment of the transfected GC cells (9). These studies indicated that GC cells provide an excellent model system in which to identify and localize the elements associated with the hGH gene that are responsible for its hormonal regulation. In the current studies we analyze the behaviour of chimaeric genes containing the 5'-flanking regions of the hGH-1, hGH-2 and hCS-1 genes fused to the bacterial chloramphenicol acetyl transferase (CAT) gene after transient transfection into GC, HeLa and Rat 2 cells. These studies indicate that the 5'-flanking regions of the hGH and hCS genes contain elements that allow their efficient expression in GC cells compared to HeLa and Rat 2 cells. These elements may be related to those that control the tissue-specific expression of the rGH gene. In addition, the 5'-flanking region of the hCS gene was shown to contain an element(s) that responds to treatment with thyroid hormone in GC cells.

EXPERIMENTAL PROCEDURES

Materials:

The plasmids containing the hGH-1 (pneo-470hGH), hCS-1 (phCS-1) and hGH-2 (phGH-2) genes are described elsewhere (3,9). The Herpes-simplex virus thymidine kinase (TK) (p-109TK) (11) gene subcloned into pBR322 was obtained from Dr. S.L. McKnight. The plasmids containing the Rous sarcoma virus promoter (RSVP) fused to the bacterial genes coding for chloramphenicol acetyl transferase (CAT) and β -galactosidase (β Gal) were obtained from Dr. M. Walker. Hybrid gene constructions were made using a CAT gene-containing plasmid (pPCAT) derived from pSV-2CAT (12); the Hind III site in pSV-2CAT was converted to a Bgl II site and the Sph I/Bam HI fragment containing the CAT gene was inserted into Sph I/Bam HI digested pBR322 (8). The Xho I/Bam HI fragment from pneo-470hGH, containing 470 bp of hGH gene 5'-flanking sequences (hGHp) and extending 2 bp downstream from the transcription initiation (CAP) site and a Sal I/Bgl II fragment from p-109TK, containing 109 bp of TK gene 5'-flanking DNA (TKp) and 53 bp of sequences downstream from the CAP site were each ligated into pPCAT, which had been digested at Sal I/Bgl II, to yield p-470hGHp.CAT and p-109TKp.CAT, respectively (8). To construct the hCS-1 and

hGH-2 gene hybrids, Hind III/Bam HI fragments, containing 496 bp of 5'-flanking sequences (hCSp and hGH-2p) from these genes and extending 2 bp downstream from their CAP sites, were ligated into a modified pPCAT vector digested with Hind III/Bgl II producing pHCSp.CAT and pHGH-2p.CAT; pPCAT was modified to accommodate these fragments by converting the Sal I to a Hind III site with synthetic linkers.

Cell Culture and Transfection:

The rat anterior pituitary tumor cells (GC), human cervical carcinoma cells (HeLa) and rat liver fibroblasts (Rat 2) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM H21) supplemented with 10% fetal calf serum (FCS). DNA transfections were performed by the procedure of Howley *et al.* (13). For experiments without hormone treatment, cells were grown to mass and split to a density of 2.5×10^6 cells per 10 cm dish 24 h before transfection. The medium was replaced immediately prior to addition of the calcium phosphate-DNA precipitate. The calcium phosphate precipitates were made in a total volume of 1 ml with 5 μ g of supercoiled CAT gene-containing plasmid and 0.5 μ g of RSVp. β Gal plasmid. The RSVp. β Gal plasmid provides an internal control for variations in the efficiency of DNA uptake among different groups of cells in each experiment. Under these conditions, the level of β -galactosidase activity was significantly above (ca. 10-fold) endogenous galactosidase-like activities. Butyrate treatment was performed as described by Gorman and Howard (14). Four h after addition of the DNA precipitate, the cells were shocked for 2 min with DMEM-20% glycerol, washed twice with phosphate buffered saline (PBS) and then fed DMEM-10% FCS supplemented with 5.0 mM sodium butyrate. The media was replaced after 24 h and the cells were harvested and counted 48 h after transfection. The cells were counted at this stage so that the extract volume, usually 0.1 ml, could be expressed as cell cytoplasm equivalents (cce). For studies involving hormone treatment, GC cells were split into DMEM-10% serum substitute (SS) (15) containing either 1% stripped FCS (STR) (16) or 1% thyroidectomized calf serum (TxCS). The medium was changed every 24 h. After 72 h deinduction the cells were immediately used for transfection as described above. The cells were glycerol-shocked, as described above, 12 h after transfection, washed with PBS and then fed DMEM-10% SS containing either 1% STR or 1% TxCS with 0.5 mM sodium butyrate and either 1.0 μ M dexamethasone (Dex), 10 nM triiodothyronine (T_3) or ethanol as a control. The cells were refed fresh supplemented medium at 24 h and harvested and counted 48 h after transfection.

CAT Assays:

Cells were lysed in 0.25 M Tris.HCl (pH 7.5) by rapid freeze-thawing; cell debris was removed by centrifugation and the extract was either stored at -70°C or used immediately for CAT and β Gal assays. Typically for CAT assays a volume of extract containing 5×10^5 cce was made up to 0.1 ml with 0.25 M Tris.HCl (pH 7.5), then 63 μ l of a mixture containing ^{14}C -chloramphenicol (6.3 nmol, 0.3 μCi) in 80 mM Tris.HCl (pH 7.5) was added followed by 10 μ l of 40 mM acetyl CoA (Sigma). Following incubation at 37°C for 6 h the reaction was stopped by adding 1 ml of ice-cold ethyl acetate and vortexing. After centrifugation, 0.9 ml of the upper phase was removed, evaporated to dryness, resuspended in 10 μ l of ethyl acetate and then separated on thin-layer chromatography (TLC) plates (American Scientific Products) in chloroform:methanol (95:5). Following autoradiography, the silica gel from the TLC plate containing both acetylated product (P) and remaining substrate (S) were scraped into separate scintillation vials and counted. Results were expressed as the ratio, P/P+S. The β Gal assay was carried out exactly as described by Nielsen *et al.* (17) using 5×10^5 cce. When CAT activity is normalized for β Gal expression ($A_{420}/24$ hours), the data are expressed as the ratio, P/ β Gal. Under these conditions the conversion of S (^{14}C -chloramphenicol) to P for -470hGhp.CAT in GC cells was typically about 10-15% and the assay was found to be linear up to at least 40-50% substrate conversion.

RNA Analysis:

Total purified cytoplasmic RNA was isolated from 10% of the cells harvested for CAT and β Gal assays according to the method of Karin *et al.* (18). RNA samples (3 μ g) in 8% formaldehyde and 6X SCC were immobilized on nitrocellulose and probed with nick-translated (19) rGH cDNA (20).

RESULTS

The -470hGhp.CAT, hCSp.CAT and hGH-2p.CAT hybrid genes were expressed under transient transfection conditions in GC cells (Fig. 1) but displayed different levels of CAT activity. The data, indicate that the hCSp.CAT and hGH-2p.CAT genes exhibit 45% and 5%, respectively, of -470hGhp.CAT gene expression (Table 1); when normalized for DNA uptake (β Gal assay) these values do not change appreciably. However, in both HeLa and Rat 2 cells the expression of all 3 hybrid genes is reduced greatly and although -470hGhp.CAT gene expression can be observed, the CAT activity is 5- to 25-fold less than that observed in GC cells (Table 1). As a control, all 3 cell types were transfected with -109TKp.CAT, RSVp.CAT and SV40p.CAT genes to ensure that the

FIGURE 1: Relative CAT activity directed by 5'-flanking sequences of hGH-related genes fused to the CAT structural gene after transient transfection in GC, HeLa and Rat 2 cells. GC (lanes a-c), HeLa (lanes d-f) and Rat 2 (lanes g-i) cells were each transfected with the -470hGhp.CAT (lanes a,d and g), hCSp.CAT (lanes b, e and h) and hGH-2p.CAT (lanes c,f and i) genes and the CAT activity was analysed after 48 h (see EXPERIMENTAL PROCEDURES).

cells were transfection competent and that the CAT gene could be expressed in HeLa and Rat 2 cells. All 3 control hybrid CAT constructions were expressed in each cell line and the relative expression of each viral hybrid gene in each cell line was comparable (Table 1). These results suggest that the 5'-flanking fragments of the hGH and hCS genes contain elements that allow for their selective expression in GC cells.

The responses of the -470hGhp.CAT and hCSp.CAT genes in GC cells to treatment with 1.0 μ M Dex or 10 nM T_3 were examined. The level of hGH-2p.CAT expression in these experiments was consistently too low to make any conclu-

TABLE 1: Relative CAT activity directed by 5'-flanking DNA of hGH-related genes and viral genes in GC, HeLa and Rat 2 cells.

	-470hGhp.CAT	hCSp.CAT	hGH-2p.CAT	-109TKp.CAT	SV40p.CAT	RSVp.CAT
GC cells:	100 (4)	45 $\pm$ 10 (4)	5 $\pm$ 1 (3)	116 $\pm$ 4 (2)	144 $\pm$ 26 (2)	573 $\pm$ 338 (2)
HeLa cells:	4 $\pm$ 1 (3)	3 $\pm$ 1 (2)	2 (1)	73 $\pm$ 30 (2)	33 $\pm$ 15 (2)	564 $\pm$ 30 (2)
Rat 2 cells:	16 $\pm$ 1 (3)	3 $\pm$ 1 (2)	3 $\pm$ 1 (2)	93 (1)	91 (1)	333 (1)

* Activity is expressed as a percentage of that observed with -470hGhp.CAT in GC cells. Values in parentheses indicate the number of experiments performed.

FIGURE 2: Effect of glucocorticoid and thyroid hormone treatment on -470hGHP.CAT and hCSp.CAT gene activity in GC cells. GC cells were transfected with -470hGHP.CAT or hCSp.CAT and then treated with 1.0 μ M dexamethasone (Dex), 10 nM triiodothyronine (T₃) or ethanol (Control). The cells were harvested and extracts were prepared for CAT and β Gal assays (see EXPERIMENTAL PROCEDURES). Panel A: Effect of hormone treatment on the endogenous rGH mRNA levels in GC cells transfected with -470hGHP.CAT (lanes a-c), or hCSp.CAT (lanes d-f); 3 μ g of total cytoplasmic RNA was isolated from a sample of harvested cells, immobilized on nitrocellulose and probed with radiolabelled rGH cDNA. Lanes a and d, Control; lanes b and e, T₃; and lanes c and f, Dex. Panel B: Expression of -470hGHP.CAT (lanes a-c) and hCSp.CAT (lanes d-f) genes in GC cells after hormone treatment. Lanes a and d, Control; lanes b and e, Dex; and lanes c and f, T₃. Panel C: Expression of -109TKpCAT gene in GC cells after hormone treatment. Lane a, Control; lane b, Dex and lane c, T₃.

TABLE 2: Relative CAT activity directed by 5'-flanking DNA of hGH-related genes in transiently transfected GC cells after treatment with glucocorticoid and thyroid hormones.

Hybrid Gene	CONTROL ^a		DEX		T ₃	
	+Butyrate ^b	-Butyrate ^b	+Butyrate ^b	-Butyrate ^b	+Butyrate ^b	-Butyrate ^b
-470hGhp.CAT:	1.0 (3)	1.0 (3)	1.3±0.1 (3)	---	0.7±0.1 (3)	0.9±0.1 (3)
hCSp.CAT:	1.0 (3)	1.0 (4)	1.4±0.1 (3)	---	4.1±1.8 (3)	5.4±2.0 (4)
-109TKp.CAT:	1.0 (2)	---	0.9	---	1.0±0.1 (2)	---

^a The CAT activity assayed after Dex or T₃ treatment is given as a fraction of the 'Control', which is arbitrarily set to 1.0. Values in parentheses indicate the number of experiments performed.

^b Experiments performed in the presence of 0.5 mM sodium butyrate as described in METHODS. For the experiments performed in the absence of sodium butyrate, the DNA as a calcium phosphate precipitate was left on the cells for 8 hrs prior to glycerol shock and the shock time was increased to 4 min.

sions about hormonal influences on its expression and consequently these studies were not pursued. Conditions were chosen under which the endogenous rGH gene mRNA levels were known to be induced by both Dex and T₃. This was monitored by examining total cytoplasmic RNA from the harvested cells by dot-blot analysis (Fig. 2A). The general pattern of responses to hormone treatment by the -470hGhp.CAT and hCSp.CAT genes in transfected GC cells are shown in Fig. 2B. Indicated in Table 2 are the fold increase or decrease in observed CAT activities averaged from additional experiments. Expression of both the hybrid hGH and hCS genes was induced slightly by treatment with Dex. However, although there was little change in -470hGhp.CAT gene activity after T₃ treatment, a significant induction (5-fold) of hCSp.CAT expression was observed. As a control -109TKp.CAT was treated identically with both Dex and T₃ and no significant response to either was seen (Fig. 2C and Table 2), demonstrating that the responsive elements were not contained either within the TK gene 5'-flanking sequences or the CAT vector.

DISCUSSION

The data (Fig. 1 and Table 1) clearly show that the chimaeric genes containing the 5'-flanking regions of the hGH and hCS genes are preferentially expressed in GC cells relative to HeLa and Rat 2 cells. The relative expression of the -470hGhp.CAT and hCSp.CAT genes in GC cells cannot be attributed to the presence of sodium butyrate, since their relative expression is

FIGURE 3: Nucleotide sequence comparison of the 5'-flanking regions of the hGH-1, hGH-2 and hCS-1 genes. The numbers indicate the approximate nucleotide positions for each of the genes; due to unequal numbers of insertion/deletions the numbers are only approximate for any given gene. The transcription initiation site occurs at nucleotide +1; the 'TATA' box is at nucleotide -30. Homologous nucleotides are indicated by capital letters and non-homologous nucleotides are indicated by lower case letters and by asterisks (*) on the line below for ease of visual comparison. The sequences for the hGH-1 and hGH-2 genes are from Seeburg (27) and the hCS-1 gene sequence is from Selby et al. (3).

the same in 5.0 mM or 0.5 mM sodium butyrate (compare Figs. 1 and 2B) and the intact hGH-1 and hCS-1 genes are expressed at comparable levels in stably transfected GC cells that have not been treated with sodium butyrate (data not shown). These results suggest that both the hGH and hCS gene 5'-flanking regions contain elements that allow for their efficient expression in GC cells. These elements might be related to elements that control the tissue-specific expression of GH-related genes in these cells. However, the expression of the hCS gene hybrid was surprising, since hCS is only known to be expressed in placental syncytiotrophoblasts and GC cells are not known to express rat chorionic somatomammotropin (rCS). Thus, the hCS gene might not be expected to contain elements that allow for its efficient expression in pituitary cells. Nevertheless, it is not clear that there is any functional relationship between hCS and rCS. The biological activities of both of these hormones are unknown. However, whereas the hCS and hGH genes have clearly

evolved from a common precursor (1,3), rCS genes have been shown to be much more related to the rat prolactin (rPr1) gene than to the rGH gene (21,22). Thus it is possible that the efficient expression of the chimaeric hCSp.CAT gene is due to the preservation of sequences present in the hGH gene 5'-flanking DNA that are capable of interacting with factors in GC cells that may be involved in the tissue-specific regulation of the rGH gene. Since the rGH, hGH and hCS genes contain about 85% nucleotide sequence homology over 150 nucleotides in the proximal promoter region (3), the elements that mediate this preferential expression in GC cells may be located in this domain.

In this regard it was of interest that the chimaeric hGH-2p.CAT gene was expressed at very low levels in GC, HeLa and Rat 2 cells. A comparison of the nucleotide sequences of the hGH-1, hGH-2 and hCS-1 genes' 5'-flanking regions (Fig. 3) indicates that the nucleotide sequence of the hGH-2 gene differs significantly between nucleotides -89 to -58 from that of the hGH-1 and hCS-2 genes. This region contains the CATAAA sequence in the hGH-1 and hCS-1 genes (CCTAAA in the hGH-2 gene) which has been shown to be an active promoter element (3). Clearly, any of the other mismatches between these genes could account for the observed variable levels of expression; however, the difference in this demonstrated promoter element is striking. The hGH-2 gene is expressed in the placenta; however, its expression is very much less than that of the hCS-1 and hCS-2 genes (P.H. Seeburg, personal communication). This difference could be related to promoter efficiency or mRNA stability. Our results are most consistent with the interpretation that elements in the proximal promoter regions of the hGH and hCS genes are required for efficient transcription in GC cells. These proximal promoter elements may be related to tissue-specific elements which control rGH gene expression. Nevertheless, it is probable that additional elements are required to control the expression of the hGH and hCS genes in human pituitary and placental cells, respectively.

Although the responses of both the hGHp.CAT and hCSp.CAT genes in GC cells to treatment with Dex was small (Fig. 2), it was reproducible. This suggests that a functional glucocorticoid responsive element (GRE) is contained in the 5'-flanking DNA of both the hGH and hCS genes. These results are consistent with the data of Robins *et al.* (23) that provided evidence that a functional GRE was present in the 5'-flanking DNA of the hGH gene. Filter binding studies also have demonstrated the presence of a glucocorticoid receptor binding region within the 5'-flanking sequences and within intron A of the hGH and hCS genes (24,25) and the GRE in intron A of the hGH gene was shown to be functional (25). Interestingly, only the GRE within intron A was capable

of generating an exonuclease III footprint after interaction with the purified rat glucocorticoid receptor, indicating that the binding affinity of the rat receptor to the GRE within intron A is greater than to the 5'-flanking sequences (24). These findings could explain the small glucocorticoid-mediated stimulation of CAT activity observed after transient transfection of the hybrid genes into GC cells.

The increased expression of the hCSp.CAT gene in GC cells in response to T₃ treatment contrasts with the hGhp.CAT gene under identical conditions, but is similar to the response of the endogenous rGH gene. In addition, the expression of a hybrid CAT gene vector containing 530 bp of rGH gene 5'-flanking DNA including its promoter was increased 1.5-fold by Dex and 2.2-fold by T₃ after transient transfection of GC cells under identical assay conditions (data not shown); Casanova *et al.* (26) have previously demonstrated the presence of a TRE within 1.7 kb of the rGH gene 5'-flanking sequences using GC cells stably transfected with a hybrid rGH/xanthine-guanine phosphoribosyl transferase gene. The extensive nucleotide sequence homology between the 5'-flanking sequences of the hGH and hCS genes (Fig. 3) suggest that only minor nucleotide changes are required to produce a change in the direction of the response to T₃ or that additional elements can modify the responses.

Although short chain fatty acids are known to influence hormone expression (28,29) it is unlikely that the 0.5 mM sodium butyrate used in this study could account for the changes in CAT activity seen with the hGhp.CAT and hCSp.CAT genes after T₃ treatment. Firstly, a negative response to treatment with T₃ was observed on hGH mRNA levels in GC cells stably transfected with the hGH gene in the absence of sodium butyrate (9). Secondly, studies in GH1 cells, a line related to GC cells, (30) indicate that less than 10% of the thyroid hormone receptors are lost after treatment with 0.5 mM sodium butyrate. Thirdly the endogenous rGH gene (Fig. 2A), the hCSp.CAT gene (Fig. 2B) and a hybrid CAT gene containing rGH 5'-flanking DNA respond positively to T₃ treatment in the presence of 0.5 mM sodium butyrate. Finally, the response of hGhp.CAT and hCSp.CAT gene expression to T₃ treatment was examined in the absence of sodium butyrate. Using a longer (4 min) glycerol shock (see EXPERIMENTAL PROCEDURES) little change in hGhp.CAT activity was seen after T₃ treatment, whereas a significant increase in hCSp.CAT expression was observed (Table 2).

The finding that the intact hGH-1 gene was negatively regulated by T₃ in

stably transfected GC cells (9) suggested the possibility that hGH gene expression may be negatively regulated by T_3 in human somatotrophs. In this study, a chimaeric hGhp.CAT gene (Table 2) showed little or no response to T_3 treatment, although in several experiments a slight decrease was detected (Fig 2B). Thus, the possibility exists that the element responsible for negative regulation of the hGH gene by T_3 is located within the gene sequences downstream of the transcription initiation site. Nevertheless, the chimaeric hCSp.CAT gene clearly contains a TRE within its 5' flanking DNA (Fig. 2B) and shares 95% sequence homology with the hGH gene 5'-flanking sequences. Furthermore, Barlow *et al.* (31) have demonstrated the interaction between the thyroid hormone receptor and the 5'-flanking sequences of both the hGH and hCS genes. Thus, it is possible that both the hGH and hCS genes contain TREs in their 5'-flanking DNA and that the presence of additional elements which modulate the T_3 response in the hGH gene account for its regulated behavior.

Our data (9, Table 2) suggest that T_3 has little effect on hGH gene expression. This is supported by the recent finding that T_3 either had no effect or slightly decreased hGH mRNA levels in primary cultures of human pituitary adenomas (R. Isaacs, P. Findell, P. Mellon, C. Wilson and J.D. Baxter, submitted for publication). Interestingly, in this latter study, T_3 treatment did result in markedly increased levels of hGH secreted into the medium. Since GH stimulation tests in hypothyroid patients usually result in blunted hGH release (32-36) and since GHRH-mediated hGH release can be (37) increased in hypothyroid patients treated with T_3 , it appears that thyroid hormone exerts a positive influence on hGH production in man. The available data are consistent with the concept that thyroid hormone control of hGH production occurs at a post-transcriptional step, and possibly at the level of hGH secretion.

In conclusion, the hGH and hCS genes contain multiple regulatory elements within their 5'-flanking sequences. The chimaeric hGH-1 and hCS-1 genes display a greater level of expression in GC cells, compared to non-pituitary derived HeLa and Rat 2 cells, suggesting that the 5'-flanking regions of these genes contain elements that may be related to the tissue-specific control elements of the rGH gene. In addition, the hGH-1 and hCS-1 genes contain GRES and TREs in their 5'-flanking DNA and the direction of the thyroid hormone response may be influenced by small nucleotide differences among the hGH and hCS gene 5'-flanking DNA regions.

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