Interaction of a Tissue-Specific Factor with an Essential Rat Growth Hormone Gene Promoter Element

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Rat growth hormone (rGH) gene expression is normally restricted to the anterior pituitary. As a model of this tissue specificity, we compared the transient expression of an rGH-chloramphenicol acetyltransferase (CAT) hybrid gene in rGH-producing rat pituitary tumor (GC) cells and in non-rGH-producing rat fibroblast (rat-2) cells. Deletion analysis of the rGH portion of this hybrid gene demonstrated that DNA sequences within 140 base pairs 5' to the rGH gene were sufficient for correct cell type-specific expression. Deletion of an additional 35 base pairs of the rGH 5'-flanking DNA resulted in a loss of expression of the transfected hybrid gene and correlated with the interaction of a putative *trans*-acting factor with this region of the rGH promoter. This factor was detectable by DNase I footprinting in a crude nuclear extract from GC cells but not from rat-2 cells. Site-directed mutagenesis of the footprint region caused complete loss of expression of a hybrid gene containing 530 base pairs 5' to the rGH gene. Thus, the interaction of this factor, which we term GC2, is likely to be essential for the tissue-specific expression of the rGH gene.

Rat growth hormone (rGH) gene expression is highly tissue specific, being expressed at 10^8 - to 10^9 -fold-higher levels in the anterior pituitary than in rat hepatoma cells (12). When the rGH gene is transfected into nonpituitary mouse fibroblast cells, an aberrant pattern of rGH transcription results (13). By contrast, rGH 5'-flanking DNA sequences are capable of promoting efficient transcription of linked genes when transfected into GC cells (2, 4), an rGH-producing rat anterior pituitary tissue culture cell line (33), but the rGH promoter appears to be inactive in nonpituitary rat and human cell lines. The transcripts in GC cells appear to be directed by DNA sequences located within 235 base pairs 5' to the mRNA initiation site (4) and to be correctly initiated (2, 4). We therefore used GC cells as a model system to identify rGH DNA sequences and associated *trans*-acting factors which are important for rGH expression in the pituitary gland. Our results suggest that a DNA sequence located between -141 and -106 relative to the mRNA start site is essential for rGH promoter activity and that there is a factor detectable in GC, but not rat fibroblast (rat-2), rat liver, or HeLa cells, which interacts with this DNA sequence.

MATERIALS AND METHODS

Construction of hybrid genes. prGH237CAT contains 237 base pairs upstream from the rGH and 7 base pairs of rGH-transcribed nucleotides (*Xho*I site, Fig. 1) linked to the bacterial gene encoding chloramphenicol acetyltransferase (CAT) (8). To simplify the constructions, the *Xho*I site was converted to a *Bam*HI site by DNA polymerase filling-in of the staggered *Xho*I end and addition of a *Bam*HI linker. The deletion of the rGH 5'-flanking DNA to position -195 (prGH195CAT) was made by oligonucleotide-directed mutagenesis of the wild-type rGH sequences at that position to generate a unique *Pst*I site (38) by using an oligonucleotide with the sequence 5'-CAAAGGCTGCAGTGGAAAG-3'.

Deletion to positions -141 (prGH141CAT) and -106 (prGH106CAT) was carried out by cleavage of the rGH 5'-flanking sequences with either *Hga*I or *Alu*I, respectively (see Fig. 1) and addition of a *Pst*I linker. Mutagenesis of the footprint region (-139 to -110) was made by using an oligonucleotide with the sequence 5'-CGCGATGTGTTT TCTTCTAGGAGCCCGGCGAACGACTACAAAGCTGTC AG-3'. The rGH-CAT hybrid gene used to test this mutation contained 530 base pairs of the rGH upstream sequences. All of the hybrid gene constructions were verified by M13 sequencing (26). The recombinant plasmid containing the Rous sarcoma virus promoter fused to the bacterial gene encoding β -galactosidase (pRSV β -gal) was obtained from M. Walker (6).

Cell culture and transfections. GC cells (33) and rat-2 cells (34) were grown in monolayer in Dulbecco modified Eagle medium (DMEM) H21 supplemented with 10% fetal calf serum (FCS). From 4 to 16 h before transfection the cells were replated to a density of 3×10^6 per 10-cm tissue culture dish. The culture medium was replaced, and the cells were transfected by the calcium phosphate technique (9) with 10 μ g of the appropriate prGH-CAT construction (Fig. 1) and 2 μ g of pRSV β -gal (as a control for DNA transfection efficiency). After 20 to 24 h, the culture medium was removed, the cells were shocked for 3 min with 15% glycerol in DMEM (24), washed twice with DMEM and 10% FCS, and incubated an additional 20 h in medium containing 1 mM sodium butyrate (7).

CAT and β -gal assays. The cells were harvested by trypsinization and disrupted by osmotic shock in 0.25 M Tris hydrochloride (pH 7.6) and three cycles of freeze-thawing. The extract was centrifuged briefly to remove cell debris and then either stored at -70°C or assayed immediately. For the CAT assay, 200 μ g of extract protein in 0.1 ml of 0.25 M Tris hydrochloride (pH 7.6) was heated to 65°C for 6 min to inactivate inhibitors of the CAT enzyme. The extract was cleared by centrifugation, and 63 μ l of a mixture containing 0.2 μ Ci of [^{14}C]chloramphenicol (40 mCi/mmol; New England Nuclear) and 0.4 mM acetyl-coenzyme A (Sigma Chemical Co.) in 80 mM Tris hydrochloride (pH 7.6) was

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FIG. 1. Schematic representation of the recombinant plasmids used in this study.

added to the supernatant. After incubation at 37°C for 3 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 recovered, evaporated to dryness, and suspended in 10 μ l of ethyl acetate. The substrate and acetylated products were separated by thin-layer chromatography (TLC) in chloroform-methanol (95:5). Following autoradiography, the silica gel from the TLC plate containing both monoacetylated products (Fig. 2, AC₁-CM, AC₃-CM) and remaining substrate (CM) was scraped into separate scintillation vials and counted. When quantitative measurements of CAT activity were made, acetylation was not allowed to exceed 50%. The β -gal assay was carried out as described by Nielsen et al. (21) with 200 μ g of extracted protein. CAT activity was normalized for β -gal expression ($A_{420}/24$ h), and the data are expressed relative to prGH237CAT activity in GC cells, which was set at 100%.

Isolation of nuclei and nuclear extract preparation. Preparation of nuclei and nuclear extraction were performed by

FIG. 2. Enzymatic assay for the ability of the various rGH-*cat* constructions to direct CAT synthesis in transfected GC cells. The position of migration by TLC of the unacetylated (CM) and monoacetylated (Ac₁-CM and Ac₃-CM) forms of chloramphenicol (CM) are marked. The recombinant plasmids used to transfect the assayed cells are noted above the appropriate lanes (237 corresponds to prGH237CAT, etc.).

the method of Strauss and Varshavsky (31). Briefly, purified nuclei were suspended to a concentration of approximately 1 mg of DNA per ml in buffer A containing 0.23 M sucrose (buffer A contains 60 mM KCl, 15 mM NaCl, 0.25 mM MgCl₂, 0.5 mM sodium EGTA, 0.5 mM spermine, 0.15 mM spermidine, 14 mM 2-mercaptoethanol, 15 mM Tris hydrochloride [pH 7.4], and the freshly added proteinase inhibitors phenylmethylsulfonyl fluoride [PMSF, 0.2 mM] and 5 μ g each of antipain, leupeptin, chymostatin, and pepstatin A [Sigma] per ml). The nuclei were pelleted at 13,000 $\times$ g for 2 min at 4°C and subsequently resuspended in one pellet volume of extraction buffer (0.35 M NaCl, 5 mM sodium EDTA, 10 mM 2-mercaptoethanol, 10 mM Tris hydrochloride [pH 7.5], and the five proteinase inhibitors at the concentrations used in buffer A). After 30 min of incubation on ice with intermittent vortexing, the nuclei were pelleted at 13,000 $\times$ g for 15 min at 4°C, and the supernatant was carefully decanted. Glycerol was added to 15% final concentration, and the supernatant was stored at -70°C until needed for footprinting assays.

DNase I footprinting. The rGH 5'-flanking sequences from -530 (*Pst*I) to +7 (*Xho*I converted to *Bam*HI) were subcloned into pUC18. The plasmid was cut with either *Bam*HI or *Bgl*II, treated with calf intestinal phosphatase, and labeled with polynucleotide kinase and [γ -³²P]ATP (specific activity, >8,000 Ci/mol). The labeled rGH fragment was subsequently released from the plasmid by restriction enzyme digestion and purified of plasmid sequences and unincorporated label by isolating the electrophoretically resolved fragment with an NA-45 membrane (Schleicher and Schuell).

Footprinting reactions were carried out after incubating approximately 5 ng of labeled DNA (5×10^3 to 10×10^3 cpm) with 30 to 100 μ g of nuclear extract (10 to 20 μ l) in a 40- μ l total volume containing 50 mM sodium phosphate (pH 6.5), 5 mM dithiothreitol, 5 mM MgCl₂, and 1.5 μ g of *Escherichia coli* DNA (sheared by sonication to lengths of 500 to 2,000 base pairs). Samples were incubated for 30 min at 20°C, after which 2 to 5 μ l of DNase I (100 μ g/ml; Worthington; freshly diluted from a 1-mg/ml stock in binding buffer) was added and the digestion was allowed to proceed for 3 to 10 min at 20°C. The amount of competitor DNA and DNase I added and the duration of digestion were determined empirically. Reactions were terminated by adding 65 μ l of a solution containing 4 M ammonium acetate, 0.1 M EDTA, and 0.5 mg of tRNA per ml to each tube. Samples were extracted once with phenol-chloroform (1:1) and once with ether and precipitated by addition of 200 μ l of isopropanol. The nucleic acid pellets were washed once in 70% ethanol, suspended in formamide sample buffer, and analyzed on an 8% polyacrylamide DNA sequencing gel (19). As molecular weight markers, the labeled DNA fragments were subjected to chemical sequencing reactions (19).

RESULTS

Deletion analysis of the rGH promoter. A series of deletion mutations in the rGH 5'-flanking DNA were constructed and analyzed for their ability to drive the expression of the bacterial *cat* gene when transfected into either GC (rat pituitary) or rat-2 (fibroblast) cells.

Transfection of prGH237CAT (Fig. 1) led to efficient expression of the *cat* gene in GC cells but not in rat-2 cells (Fig. 2 and Table 1), while the cotransfected RSV β -gal plasmid was efficiently expressed in both cell lines (data not shown). Primer extension analysis indicated that transcrip-

tion of prGH237CAT initiated at the correct position (data not shown), conforming earlier reports (2, 4). Deletion of the rGH 5'-flanking sequences to either nucleotide position -195 (prGH195CAT) or -141 (prGH141CAT) led to a gradual decrease in expression of CAT activity in GC cells (Fig. 2, Table 1), although levels remained easily detectable in GC cells and undetectable in rat-2 cells. These data suggest that the factor(s) conferring tissue-specific expression of rGH in GC cells lies within 141 base pairs from the start site of transcription.

Deletion of an additional 35 base pairs of the rGH 5'-flanking sequences (prGH106CAT) led to a drop in CAT expression in transfected GC cells to the level seen with a vector containing the *cat* coding sequences but no promoter (compare 106 and Δ , Fig. 2 and Table 1), strongly suggesting that a crucial element for rGH expression resides in or near these deleted sequences.

Expression-linked rGH promoter sequences bind a nuclear factor detected only in rGH-producing cells. To determine whether we could correlate the data obtained by transient expression with the interaction of *trans*-acting factors with the rGH 5'-flanking DNA, we performed DNase I footprinting assays (Fig. 3). When the rGH 5'-flanking sequences were incubated with DNase I, a specific pattern of cut sites was generated (Fig. 3, lane 3). When the DNA fragment was first incubated with a nuclear extract from GC cells before DNase I cleavage, the pattern of digestion seen with naked DNA was very specifically modified (compare lanes 3 and 4, Fig. 3A), as characterized by a general protection of the region between -111 and -142 on the noncoding strand (Fig. 3A, lane 4) and between -106 and -134 on the coding strand of the rGH promoter (Fig. 3B, lanes 6 to 8). This protected region was absent when the rGH DNA fragment was incubated with the same amount of protein prepared in the same manner from rat-2 cell nuclei, a cell line which does not express either the endogenous or transfected rGH (Fig. 3A, lane 5). Similarly, no footprint was obtained in this region from nuclear extracts of non-rGH-expressing HeLa or primary rat hepatocyte nuclei, although the same extracts gave footprints at other positions in rGH and other genes (data not shown). Thus, this factor, which we designate GC2, not only binds a DNA sequence that is crucial for expression of the transfected rGH-CAT constructions in GC cells, but also is undetectable by this assay in non-rGH-producing cells.

Specific mutation of the footprint region abolishes rGH promoter activity. To directly assess the importance of the GC2 factor interaction site, this site (bases -139 to -110 with respect to the RNA initiation site) was mutated by oligonucleotide-directed mutagenesis (38); 530 base pairs of either the native rGH 5'-flanking DNA or the same DNA

FIG. 3. DNase I footprinting assay for specific factor interactions in the rGH 5'-flanking sequences. (A) Noncoding strand, labeled at *Bam*HI (+7; see Fig. 1). Lanes 1 and 6 contain G, and lanes 2 and 7 contain G+A sequencing ladders of the labeled template, which were run in parallel as size markers. Lanes 3 to 5 show the cleavage pattern of the labeled DNA template obtained by digestion with DNase I either in the absence of added nuclear extract (lane 3) or after preincubation with 100 µg of nuclear extract from either GC (lane 4) or rat-2 (lane 5) cells. (B) Coding strand, labeled at *Bgl*II (+237, see Fig. 1). Lanes 1 to 4 contain G, G+A, C+T, and C sequencing reactions, respectively, for the labeled template. Lanes 5 to 8 show the cleavage pattern obtained with DNase I either in the absence of nuclear extract (lane 5) or after incubation with 30 µg (lane 6), 60 µg (lane 7), or 90 µg (lane 8) of GC cell nuclear extract. The coordinates for the limits of the protected region are marked at the right. (C) Nucleotide sequence and coordinates for the region of the rGH promoter protected from DNase I digestion by binding of GC2.

containing the mutant GC2 binding site was linked to the *cat* gene and used to transfect GC cells. The results suggest that specific mutation of the GC2-binding site reduces rGH promoter efficiency to a level equivalent to the complete deletion of the promoter (Fig. 4). This result indicates that the GC2 factor interaction site contains an element(s) which is essential for rGH promoter activity.

DISCUSSION

Tissue-specific expression of the transfected rGH gene has previously been demonstrated to be directed by DNA sequences 5' to the gene (2, 4) and within 235 base pairs of the transcription start site (4). Our present studies were undertaken to identify the elements within this region of the rGH-flanking DNA which are responsible for expression of the gene exclusively within the pituitary gland. We have found that tissue-specific activity of the rGH promoter is maintained with only 141 base pairs of 5'-flanking DNA. Within these 141 base pairs, we have identified a binding site for a nuclear factor which we detected only in rGH-producing culture cells. We refer to this factor as GC2. When the GC2-binding site was either deleted or mutated, rGH-*cat* gene expression was completely eliminated. Since the GC2-binding activity was found only in nuclei of rGH-producing cells, we feel it is likely that its function is essential for determining the correct tissue specificity of rGH gene expression, although other factors acting either upstream or downstream of the GC2 interaction site may also contribute to this specificity.

TABLE 1. Relative expression of the prGH-*cat* constructions in GC and rat-2 cells^a

Plasmid	Expression in GC cells (% of control)	No. of expt	Expression in rat-2 cells (% of control)	No. of expt
prGH237CAT	100	9	<1	2
prGH195CAT	40.6	9	<1	2
prGH141CAT	27.6	8	<1	2
prGH106CAT	5.7	9	<1	2
pΔCAT	7.2	9	<1	2

^a The expression of prGH237CAT in GC cells (24 to 40% conversion in separate experiments) was set to 100% (control) for each individual experiment. See Materials and Methods for details.

FIG. 4. Mutation of the GC2 interaction site abolishes rGH promoter function. Nonconservative transversion mutations were introduced in the DNA sequences of the rGH promoter between -139 and -110 relative to the RNA start site by site-directed mutagenesis. The expression of *cat* gene hybrid constructs containing either the native rGH promoter (-530 [native]), the same promoter containing the mutation (-530 [-139, -110]), or no rGH promoter (Δ) was compared after transient expression in GC cells. The vector and assay systems are described in the text and in the legends to Fig. 1 and 2. In four experiments, the expression from the native rGH promoter (2.2 to 23.5% conversion, set to 100%) was diminished by the mutation to an average relative level of 4.6%, which is similar to the average relative level of 9.3% for the promoterless construction.

Expression of the endogenous rGH gene has previously been correlated with an unmethylated CGCG sequence located 144 base pairs 5' to the mRNA start site of the rGH gene (32). This site is located within 7 base pairs of the 5' border of the observed GC2-binding site, which raises the possibility that these two phenomena are related. Such methyl substitutions, which would project into the major groove of B-DNA (25), might affect the binding of the GC2 factor. Alternatively, the binding of GC2 might block methylation from occurring. Further work on the effects of methylation at this site is needed.

We have previously identified another pituitary cell-specific factor (GC1) which binds to the rGH promoter at a specific site downstream of GC2. Unlike the sequence protected by GC2, however, those protected by GC1 do not seem to be essential for the function of the rGH promoter in GC cells (D. F. Catanzaro, B. L. West, J. D. Baxter, and T. L. Reudelhuber, *Mol. Endocrinol.*, in press). Recently, White et al. (36) described two chromatin proteins (NP44 and NP48) in both GC cells and a related cell line, GH₃, which bound specifically to rat prolactin 5'-flanking sequences located more than 600 base pairs upstream of the mRNA start site. Because the actual sites of interaction were not identified in that study, we do not know if these prolactin gene-binding factors are related to GC1 and GC2. Since the prolactin gene is also expressed in pituitary cells (3, 11), it is possible that these two genes may use some common transcription factors. A computer search (17, 18) for regions within the rat prolactin 5'-flanking DNA which are homologous to the rGH GC2 interaction site revealed several possible sites, but none with homology greater than 51% over the 35 nucleotides protected by GC2. Footprinting studies of the rat prolactin gene should help to determine whether this gene does utilize any of the factors which

regulate rGH expression. A computer search (17, 18) for other eucaryotic genes with homologies to the 35 base pairs of the GC2 footprint failed to show any sequences with homologies as great as within the growth hormone gene family (5, 27, 28, 37). It is not yet possible to evaluate the importance of sequences having much less homology due to the lack of information about which bases within the GC2 interaction site are critical for GC2 to bind and whether there is actually more than one factor which binds at this site.

Although the GC2-binding region appears to be essential for rGH promoter activity, it is noteworthy that substantial decreases in rGH-*cat* expression were observed with the deletions of DNA sequences upstream of -141 (Fig. 2, Table 1). These decreases imply that rGH expression is regulated by multiple factors. Other mammalian genes which are expressed in a tissue-specific manner use a combination of factors to regulate their tissue-specific expression (6, 10, 14, 22, 23, 29, 35). Also, it is known that the rGH gene is regulated at the transcriptional level by multiple effectors such as thyroid hormone, glucocorticoids (30), and cyclic AMP (1). Indeed, it has recently been demonstrated that at least one component of the thyroid-responsive element for the rGH gene resides within 235 base pairs 5' of the RNA start site (4) and upstream of position -183 (15) and that the sequences upstream of -183 have a substantial effect on the basal level of rGH expression (16, 20). It is likely that other factors interact with the sequences between -237 and -141, but we have not yet been able to identify such factors with crude nuclear extracts and DNase I footprinting. Characterization of the sites of interaction of other possible effectors in the rGH promoter may require some purification of the nuclear extracts and perhaps the use of a more sensitive binding assay (29).

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