Glucocorticoids Regulate the Expression of a Rat Growth Hormone Gene Lacking 5' Flanking Sequences*

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Rat growth hormone (rGH) gene expression is regulated by glucocorticoids in vivo and in cultured pituitary cells. After the co-transfer of a plasmid containing the rGH gene into mouse L-cells (with or without the simian virus 40 enhancer), little or no normal rGH mRNA is produced. Instead, the predominant rGH gene transcripts are about 0.75 kilobase pairs (kb); these lack the first two exons of the rGH but possess a 3' end that terminates accurately. Nevertheless, the levels of these transcripts are increased by glucocorticoids. When all of the rat sequences 5' to an XhoI site located 7 nucleotides downstream from the rGH gene physiological cap site are deleted and the mutant gene introduced into L-cells, the transfected cell lines still produce the 0.75-kb transcripts; however, in addition, these cells produce a more abundant 1.1-kb mRNA that has a 3' terminus similar to that of rGH mRNA, but whose 5' termini begin in the region of, but not at, the initiation site used in pituitary cells. Both of these transcripts are increased 3- to 5-fold by 1 μM concentration of the glucocorticoid dexamethasone. These data indicate that 1) deletion of all of the rGH gene 5' flanking sequences allows formation of approximately full length transcripts and 2) sequences containing information for regulation of rGH gene expression in L-cells by glucocorticoids are contained in the structural portion of the gene and/or the 3' flanking sequence.

Rat growth hormone gene expression is regulated by thyroid hormone and glucocorticoids in the somatotrophs of the anterior pituitary as well as in cultured pituitary tumor cells (1-3). Both classes of hormones independently as well as synergistically increase growth hormone secretion and the changes in synthesized protein correlate with the levels of mRNA (4-6). Moreover, both classes of hormone stimulate gene transcription (3); however, the increase in rGH gene transcription in response to the glucocorticoid may not account fully for the observed change in rGH mRNA levels (3).

Recently, several glucocorticoid-regulated genes have been transferred into responsive cells and shown to be regulated by dexamethasone, a synthetic glucocorticoid (7-11). In some cases, such as the mouse mammary tumor virus (12-14) and the human metallothionein IIA gene (15), the regions of DNA responsible for conferring sensitivity to steroids have been mapped and are the same as those sequences that bind the steroid receptor. In each case, the regulatory regions were found to be adjacent and 5' to the RNA polymerase II promoter of the steroid-responsive gene. The rat growth hormone gene has been introduced into the mouse L-cells that are defective in thymidine kinase by co-transfer with the selectable herpes simplex virus TK gene (16). All of the cell lines that expressed the transfectected gene were found to have a predominant polyadenylated transcript that lacks the first two exons and is about 300 nucleotides shorter than the physiological rGH mRNA. However, in all cases, the levels of rGH mRNA in these cells were increased 3- to 5-fold after treatment with dexamethasone. The data of the present report suggest that: 1) sequences downstream from the rGH gene physiological initiation site are sufficient for regulation by glucocorticoids; 2) glucocorticoids increase the levels of rGH gene transcripts initiating at sites other than the normal rGH gene promoter; and 3) deletion of 5' flanking sequences allows expression of approximately full length rat growth hormone mRNA.

EXPERIMENTAL PROCEDURES

Materials—The plasmids containing the rat growth hormone gene, pRGHeh5.8, and cDNA are described elsewhere (21, 22). The plasmid which contains the SV40 enhancer region (prC), prepared by cloning the SV40 HindIII C fragment into the HindIII site in prGHeh5.8 approximately 1.5 kb 3' to the growth hormone termination site, was a gift from Emily Slater (University of California, San Francisco). pHSV106, which contains the HSV-TK gene, was from Bethesda Research Laboratories.

Restriction enzymes were from New England Biolabs, Inc. or Bethesda Research and were used as recommended by the supplier. Bacteriophage T, DNA ligase and the large fragment of DNA polymerase I were obtained from Bethesda Research Laboratories. Calf alkaline phosphatase was from Boehringer Mannheim. Reverse transcriptase was obtained from Life Sciences, Inc. T4 DNA polymerase and T4 polynucleotide kinase were obtained from P-L Biochemicals. [γ-32P]ATP (7000 Ci/mol) was from ICN and [α-32P]deoxynucleotide triphosphates (3000 Ci/mol) were from Amersham.

Cell Culture—Mouse LTK⁻ cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM-H16; Cell Culture Facility, University of California, San Francisco) supplemented with 10% fetal calf serum (HyClone) and 100 units/ml of penicillin and 50 μg/ml streptomycin. The rat pituitary tumor cells (lines GH and GC) were grown in similar media, except that it contained 4.5 g/l of glucose. All cells were grown in a 10% CO₂ atmosphere.

Transfection—Cell transfections were performed according to Wiggs et al. (16). LTK⁻ cells were exposed to 1 ml of a calcium phosphate
precipitate containing 1 μg of circular plasmid, 100 ng of pHSV196, and 10 μg of salmon sperm DNA. After 12 to 16 h, TK\(^{+}\) transformants were observed in about 2 weeks and grown into mass culture for further study.

**Preparation and Characterization of Nucleic Acids**—High molecular weight DNA was isolated from nuclei prepared with Nonidet P-40 (17). Nuclei were washed and then treated with proteinase K overnight. The DNA was extracted with phenol/phenol chloroform and extensively dialyzed against 1 mM EDTA, 10 mM Tris-HCl pH 8.0 (TE) (17). DNA was cleaved with the appropriate restriction enzyme, size-fractionated by agarose electrophoresis, and transferred to nitrocellulose filters (Schleicher and Schuell RAKS). Hybridization was performed according to the recommendation of Schleicher and Schuell in the presence of 50% formamide, 0.04% Ficoll, 0.04% polyvinylpyrrolidone, 0.04% bovine serum albumin, 0.1% sodium lauryl sulfate, 200 μg/ml salmon sperm DNA 5 X SSPE (1 X SSPE is 0.18 M NaCl, 10 mM NaPO\(_4\), pH 7.7, 1 mM EDTA), 10% dextran, and about 5 ng/ml probe at 42 °C for 24 to 48 h.

Total cytoplasmic RNA was isolated after phenol/chloroform extraction of cytosol according to Maniatis et al. (17) except that the sucrose layer was omitted in preparation of nuclei. Total RNA was isolated by the method of Cathala et al. (18). RNA was denatured with formaldehyde, size-fractionated by agarose electrophoresis, and transferred to nitrocellulose membranes (Schleicher and Schuell, PHT9). Hybridization conditions were the same as those used with DNA (see above).

Probes labeled on a single strand were prepared using the bacteriophage T4 DNA polymerase technique (19). The appropriate fragment was isolated from low melting point agarose (Seaplaque, FMC Corp.) and purified with NACS Prepac columns (Bethesda Research Laboratories, Inc.).

Nitrocellulose filters were exposed to Kodak XAR-5 film at -70 °C with intensifying screens (Dupont, Cronex). S\(_{1}\) endonuclease mapping was performed essentially according to Weaver and Weissman (20). Plasmid DNA was cleaved with appropriate restriction enzymes and labeled with either T4 polynucleotide kinase (for mapping the 5' end) or T4 DNA polymerase (for mapping the 3' terminus). Fragments were either isolated from low gelling agarose as above or from polyacrylamide gels by extraction into 0.5 M NaCl. Twenty μg of total RNA or 2 μg of poly(A\(^{+}\)) RNA was incubated with the end-labeled fragment for 5–12 h in 80% formamide, 0.5 M NaCl, 1 mM EDTA, and 50 mM Pipes, pH 6.4. Digestion was performed at 30 °C for 75 min using about 1000 units/ml of S\(_{1}\) nuclease (Sigma). DNA-RNA hybrids were denatured and electrophoresed on urea-acrylamide gels which were dried and exposed to Kodak XAR-5 film either at room temperature or at -70 °C with intensifying screens.

Primer extension analysis was performed using a 41-deoxynucleotide oligomer synthesized on a Applied Biosystems 380A DNA synthesizer. The oligonucleotide was purified by polyacrylamide gel electrophoresis and DE52 chromatography. For the annealing reaction, 2 pmol of "P-5' labeled primer was mixed with 5 μg of poly(A\(^{+}\)) RNA or 20 μg of total RNA in 0.4 M KCl, 10 mM Tris-HCl pH 8.3, 1 mM EDTA, and 5 mM vanadyl ribonucleotide complex (BRL) in 0.02 ml, heated to 65 °C, then incubated at 25 °C for 2 h. One-third of the annealed mixture was diluted and the hybrid was extended for 1 h at 42 °C in the presence of nonradioactive deoxynucleotide triphosphates and reverse transcriptase, extracted with phenol/chloroform, electrophoresed, and visualized as described above.

**RESULTS**

Fig. 1 shows an analysis of rGH gene transcripts in L-cells transfected with the rGH gene contained in a plasmid into which the SV40 enhancer sequences have been inserted approximately 1.5 kb 3' to the rGH gene transcription termination site. The normal 1.1-kb growth hormone mRNA was not found; instead, the predominant rGH gene transcript is 0.75 kb (Fig. 1). Treatment of the cells with 1 μM dexamethasone results in a 3- to 5-fold increase in the levels of these transcripts. These results are similar to those previously obtained when the transfected rGH gene was not linked to a viral enhancer (11). The specificity of this response is documented by the fact that the steroid had no effect on the levels of thymidine kinase mRNA (data not shown); this result is in agreement with our previous findings (11) and those from other laboratories (9). As previously reported, single-stranded nuclease protection experiments suggest that the small transcript was either initiated in the second intron or generated from splicing of a transcript initiated further upstream with an acceptor site in the intron. If the former were the case, deletion of all of the rGH gene 5' flanking sequences might not affect expression of the abbreviated transcript. To test this, the plasmid prGH\(_{Apro}\) was constructed. This was deleted in all rat sequences 5' to the Xhol site located 7 nucleotides downstream from the CAP site used in pituitary cells, but contained virtually the entire rGH pre-mRNA sequence and about 1.5 kb of 3'-flanking DNA (Fig. 2A). prGH\(_{Apro}\) was co-transfected with the HSV-TK gene into mouse L-cells and single colonies surviving in HAT medium grown to mass culture. A Northern blot in which RNA from two of these lines was hybridized to a \(^{32}\)P-labeled noncoding strand of the rGH cDNA localized two RNA species, one with a size of 0.75

**FIG. 1. Effect of dexamethasone on rGH gene transcripts in L-cells transfected with prC.** Total cytoplasmic RNA (20 μg) isolated from Ltk\(^{-}\) cells transfected with prC, after incubation for 48 h with or without 1 μM dexamethasone, was run on a 1.5% agarose gel along with 5 μg of total GH\(_{2}\) cell RNA. The rGH cDNA probe was prepared by cleaving prGH-1 with EcoRI, digesting with T4 DNA polymerase such that approximately 800 nucleotides were removed, and then adding deoxynucleoside triphosphates including [\(^{32}\)P]-dATP and [\(^{32}\)P]-dCTP. The plasmid was then cleaved with HindIII, the labeled 800-bp fragment was isolated and used as a hybridization probe. Markers (lane M) are HaeIII-cleaved eX174 DNA labeled with T4 DNA polymerase and [\(^{32}\)P]-dCTP.
kb and another more abundant transcript of 1.1 kb (Fig. 2B). The levels of both transcripts increased after treatment with dexamethasone. Fig. 2C shows a blot of another transfected line after exposure of the filter to x-ray film for two different time periods. In the 2-day exposure, regulation of the large transcript is better visualized in the longer exposure. All of the six independent clones of transfected L-cells that expressed the deleted rGH gene produce 0.75- and 1.1-kb transcripts whose levels were increased by the glucocorticoid; however, the absolute levels of expression varied among the cell lines.

In order to study the organization of the rGH gene fragments in transfected L-cells, high molecular weight DNA from several transfected clones as well as GH2 and GC cells was prepared and used for Southern blot analysis. In the absence of restriction enzyme digestion, Southern blots show all hybridizable material in the 20–50-kb size range, indicating integration of plasmid into high molecular weight DNA (data not shown). After digestion with Pst I, L-cells produce two hybridizing bands of approximately 900 bp and 6200 bp that presumably represent the endogenous mouse gene (Fig. 3). Four different prGHΔpro transfected lines produced consistent patterns: in addition to bands from the mouse genes there are the three bands of 850, 1250, and 3025 bp that are much greater than those from GH2 and GC cells implying the presence of multiple rGH gene copies in the transfected cells (Fig. 3). There are also a few minor bands that differ among transfected cell lines that may represent either the junctional rGH gene sequences flanking the concatamer or integration of partial fragments of the transfected gene. Thus, the presence of major bands predicted from digestion by Pst I of prGHΔpro suggests that gene sequences are integrated into high molecular weight DNA in the form of grossly unarranged “head-to-tail” concatamers with copy number differing among the cell lines. To understand better the nature of the two transcripts, specific probes from the 5’ and 3’ ends of the rGH gene were used. A probe that contained most of the fifth exon and part of the fourth intron hybridized to both the large and small mRNAs from prGHΔpro transfectants (Fig. 4). As expected, the same probe also hybridized to the 1.1-kb rGH mRNA from GH2 cells and the 0.75-kb rGH gene transcript from L-cells transfected with prGHeh5.8. By contrast, hybridization of a labeled DNA probe containing the first two exons and the first intron of the rGH gene to a similarly run Northern blot, identified the piuatory transcript and the 1.1-kb species in prGHΔpro transfectants, but did not the 0.75-kb mRNA in cells transfected with either the prGHΔpro or the intact rGH gene. These data suggest that the structure of the 3’ but not the 5’ end of the 0.75-kb rGH gene transcripts was preserved after transfer to mouse L-cells.

S1 nuclease protection experiments were also performed (20). Use of a 190-bp fragment spanning the physiological termination site 3’ end-labeled with bacteriophage T4 DNA polymerase and total RNA prepared from L-cells, GH2, and L-cells transfected with either prGHeh5.8 or prGHΔpro verified that all of the rGH gene transcripts utilized the polyadenylation site predicted (22) by the sequence of the rGH cDNA (Fig. 5). To map the 5’ end of the transcripts, a DNA fragment from the prGH cDNA beginning in exon III and extending into the flanking pBR322 sequence was 5’-labeled with bacteriophage T4 polynucleotide kinase (Fig. 6). This
probe lacks the first 38 nucleotides of the 5'-nontranslated region of pituitary rGH mRNA and cannot be used to map start sites upstream from the region. However, it is useful for evaluating the accuracy of splicing as well as the sum of all mRNA species originating upstream from the sequences included in the cDNA probe. As can be seen in Fig. 6, an S1 nuclease protection experiment with this probe generates the same protected fragment for the RNA from prGHΔpro transfectants as for that from GH3 cells. Furthermore, the intensity of this band is increased in RNA from cells treated with dexamethasone. These data show that for the prGHΔpro transfectants: 1) the first two introns are spliced accurately, 2) the transcripts start upstream from the beginning of the cDNA, and 3) the levels of these transcripts are increased in response to steroid. In order to more precisely identify the initiation of transcription, a primer extension experiment was performed. An oligonucleotide complimentary to residues 80 to 120 of the normal predominant rGH transcript found in pituitary cells (21) was 5'-end labeled, annealed to either total or poly(A)+ RNA from transfected cells, incubated in the presence of nonradioactive deoxynucleotide triphosphates and reverse transcriptase, and separated by denaturing polyacrylamide gel electrophoresis. As shown in Fig. 7, RNA from GH3 provided a template for an extension product exactly the predicted 120 nucleotides. In contrast, poly(A)+ RNA from L-cells transfected with prGHΔpro directs the synthesis of several products (noted by open arrows) approximately, but not precisely, the same length as that formed in GH3 cell RNA. These same species are present but less abundant in total RNA as would be expected of extension products dependent on poly(A)+ mRNA. There are also some less intense, greater molecular weight products. None of these bands was found when primer was annealed to and extended in the presence of nontransfected L-cell RNA (Fig. 7, lane L). Thus, L-cells transfected with the promoterless rGH gene contain transcripts beginning in the region of but not precisely at the physiological initiation site.

One possibility was that the near full length rGH transcripts found were being directed by bacterial vector sequences fortuitously located near the eukaryotic 5' sequence. In order
to test this, another recombinant plasmid containing the rGH gene in the opposite orientation was constructed. First, the Xhol site in prGHeh5.8 located 7 base pairs downstream from the physiological CAP site was converted to a BamH1 site using T1 polymerase and synthetic linkers. Then the rGH-containing BamH1 fragment was cloned into the BamH1 site of pBR322 producing the plasmid prGHΔpro(−) with the rGH gene in the opposite orientation compared to prGHΔpro (Fig. 8A). prGHΔpro(−) was co-transfected with pHSV106 into L-cells, the cells surviving in HAT were pooled, and total RNA was prepared with and without prior dexamethasone treatment. A Northern blot (Fig. 8B) using a probe prepared from the rGH cDNA revealed the same two bands seen in prGHΔpro transfected cells and the abundance of both products was increased after treatment with dexamethasone.

**DISCUSSION**

The current studies have focused on the expression and regulation of the rGH gene deleted in the 5' flanking DNA and the first 7 nucleotides of the 5' untranslated regions after its transfer to mouse L-cells. Clearly, information enabling dexamethasone to regulate rGH gene expression is contained within the gene's structural portion or 3' flanking sequences as evidenced by the hormone responsiveness of fibroblasts transfected with a rGH mutant gene deleted in all 5' flanking sequences. That the transfectant lines do indeed contain the unarranged prGHΔpro plasmid is indicated by Southern blot analysis (Fig. 3B). In particular, the 3025-bp PstI fragment seen is not contained in the native rGH containing plasmid or in rat pituitary DNA, since it is generated by the ligation of the "promoterless" structural rGH gene to pBR322 (Fig. 3A). The differences in copy number as well as the variance in minor hybridizing bands of the Pst1-digested DNA indicate that the cell lines do indeed represent independent clones (23). Since glucocorticoids regulate the rGH transcripts in a number of different transfectants (Fig. 2), the necessary information must be present on the "promoterless" rGH gene plasmid. These results provide evidence for a mode of regulation alternate to the many reports suggesting a site of glucocorticoid actions in the 5'-flanking DNA (12-15).

**FIG. 5. S1 endonuclease mapping of termination sites.** Twenty μg of total RNA was hybridized to the 32P-end-labeled 190-bp PvuII fragment from prGHeh5.8 which spans the polyadenylation site. The hybrids were treated with S1 nuclease, separated on a polyacrylamide sequencing gel, and exposed to film. Lanes are labeled as in Fig. 4. RNA isolated from untransfected L-cells was also used. Markers are pBR322 cleaved with HpaII and labeled with [α-32P]dCTP by the Klenow fragment of DNA polymerase. The rGH gene exons are shown as darkened boxes and labeled E3, E4, and E5 with the remaining gene segments as open boxes or wavy lines.

**FIG. 6. S1 endonuclease mapping of the 5' ends.** Conditions are as in Fig. 5, except that transfactant cells were incubated with and without 1 μM dexamethasone for 48 h and the probe was the 5' end-labeled 773-bp HpaII fragment of the rGH cDNA plasmid. This fragment contains sequences from exons 1 to 3 and 491 bp of pBR322, but lacks 38 bp corresponding to the 5' end of the transcripts found in pituitary cells.
Fig. 7. Primer extension mapping of the 5' end. The 5' end-labeled primer (described in text) was annealed to RNA, exposed to reverse transcriptase in the presence of nonradioactive deoxynucleotide triphosphates, separated on a 6% polyacrylamide sequencing gel, and exposed to film. The bottom of the gel containing the primer has been cut off. Lanes and markers (M) as in Fig. 5; prGHΔproA+, 0.75 µg of poly(A+); prGHΔpro total, 3 µg of total RNA from L-cells transfected with prGHΔpro; L, 3 µg of total RNA from the same cell line; GH3, 3 µg of total RNA from GH3 cells. The size of the expected extension product is indicated by a solid arrow and several abnormal products by open arrows.

Results of studies on the control by glucocorticoids of expression of mouse mammary tumor virus and the metallothionein gene have suggested a general model for gene regulation by steroid hormone (12-15, 24). The steroid-receptor complex initiates the events leading to increased gene transcription by binding to a specific region distinct from the promoter and 5' to it. It has been difficult to determine if the 5' flanking region of the rGH genes confers glucocorticoid sensitivity in fibroblasts since the rGH promoter is essentially inactive in L-cells either as part of the intact gene (11) or fused to the TK structural gene. The present data indicate that glucocorticoids can regulate rGH gene expression by a mechanism different than metallothionein or mouse mammary tumor virus. Two alternative models might explain this difference: 1) located within the gene or its 3' flanking sequence is a regulatory region to which receptor-steroid complex binds inducing an increase in transcription from an upstream promoter (analogous to the enhancing region in the second intron of the mouse immunoglobulin genes (25-27)); or 2) the regulation is at a post-transcriptional site by a mechanism dependent upon information encoded within the rGH mRNA. The latter model might involve either direct binding of receptor-steroid complex to a rGH gene transcript or induction of an as yet unidentified regulatory molecule. A critical difference between these two hypotheses is the kinetics of induction and we are currently attempting to measure directly rates of transcription and mRNA degradation in transfected cells.

Quite surprisingly, Northern blot analysis of all expressing lines transfected with prGHΔpro revealed apparently full length transcripts while transfer of the intact gene into L-cells yields only the smaller 0.75-kb transcript (Fig. 2). The human growth hormone gene transferred into the same fibroblast line produces a correct size transcript, although the precise initiation site has not been mapped (9). Although it has been proposed that the absence of physiological initiation

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2 M. J. Birnbaum and J. D. Baxter, unpublished observations.
in rat growth hormone transfectants is due to a lack of tissue-specific factors (11), the difference between transcription of the rat and human genes remains unclear. Equally perplexing is how deletion of all putative regulatory regions allows expression of approximately full-length transcripts. It is unlikely that the transcripts originate from eukaryotic promoter-like sequences in pBR322, since there are no TATAA-like sequences on the appropriate strand in this region of the plasmid (28) and similar-size transcripts are formed in transfectants with the rGH gene in the opposite orientation. It has not been ruled out that the transcripts initiate further upstream and are spliced to multiple acceptor sites in the regions of the XhoI site. However, the S1 protection experiments indicate that at least the first two introns are processed accurately and the transcript terminates properly in the transfected cells (Figs. 5 and 6). Thus, the simplest explanation at this time is that some element within the gene is influencing upstream initiation which, in the absence of a TATAA box, occurs at multiple sites and is suppressed in fibroblasts when sequences normally flanking the rGH gene are present. Further studies are now in progress to understand the in vivo transcription of the “promoterless” gene as well as its regulation by hormone.

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