Identification of glucocorticoid-responsive elements that control transcription of rat glutamine synthetase

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Chandrasekhar, S., Wiley W. Souba, and Steve F. Aboouwer. Identification of glucocorticoid-responsive elements that control transcription of rat glutamine synthetase. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L319–L331, 1999.—Basal expression of glutamine synthetase (GS) is very low in rat lung and muscle and remarkably enhanced by glucocorticoid hormones during trauma and catabolic states. Although this response is believed to be transcriptionally regulated, the genetic elements responsible for tissue-specific glucocorticoid induction of GS expression have not been identified. A rat lung epithelial cell line (L2) and a glucocorticoid receptor-deficient human prostate cancer cell line (PC3), together with GS reporter gene constructs, were utilized in gene transfer experiments to identify two regions within the rat genomic clone gGS3 that imparted dexamethasone (Dex) responsiveness to both the homologous GS promoter and the heterologous herpes simplex virus thymidine kinase promoter in glucocorticoid receptor-dependent fashions. One region lies nearly 6 kb upstream of the GS transcription initiation site, and the other lies within the first intron of the GS gene. Dex responsiveness was localized to a 325-bp fragment of the intron region containing a canonical glucocorticoid response element and to a 225-bp fragment of the far-upstream region containing three separate glucocorticoid response element half-sites. The GS promoter exhibited relatively high basal activity that was repressed by inclusion of the far-upstream or the intron glucocorticoid-responsive region. Dex treatment negated this repression. A model is suggested in which the glucocorticoid-receptor unit causes derepression of lung and muscle GS transcription during trauma and catabolic states.

During catabolic states, visceral utilization of the amino acid glutamine is heightened. To meet this enhanced demand and maintain plasma levels, glutamine production by lung and muscle is enhanced (for a review, see Ref. 2). In the rat, expression of glutamine synthetase (GS) is increased in the lung and muscle during a number of catabolic states, with induction of GS mRNA levels by 400–700% (3, 6, 7, 9–11). Induction of GS gene expression in both the lung and muscle of endotoxemic rats is largely dependent on adrenal-derived hormones (36, 37). Likewise, GS expression is markedly induced specifically in rat lung and muscle after injection of the synthetic glucocorticoid hormone dexamethasone (Dex) (1). Dex alone increases GS expression severalfold in a number of mammalian cell lines, including rat lung cells, by a direct glucocorticoid receptor (GR)-mediated mechanism (see Refs. 4, 5 and references therein). Thus GS ranks as one of the most remarkably glucocorticoid-inducible mammalian genes.

Surprisingly, the genetic elements responsible for the glucocorticoid inducibility of mammalian GS have not been determined. Developmental and hormonal regulation of GS expression have been extensively studied in the embryonic chicken retina (42), and upstream genetic elements that contribute to the glucocorticoid inducibility of avian GS have been identified (52). In contrast to the chicken GS promoter, which has eight canonical glucocorticoid response elements (GREs), previous examination of 2.1 kb of the rat GS promoter revealed a single element with weak sequence similarity to the canonical GRE located 406 bases 5′ of the transcription start site (51). However, the role of this element in the glucocorticoid-mediated induction of rat GS transcription is unclear (51). Thus, although GS expression is remarkably inducible by glucocorticoid hormones, the genetic elements responsible for this regulation are completely unknown.

In this study, cultured rat lung epithelial cells (L2), as well as GR-deficient human prostate cancer cells (PC3), were utilized to test regions of the rat GS gene for the ability to confer glucocorticoid regulation of transcriptional activity. Two regions have been identified: one far upstream from the transcriptional start site of GS and one within the first intron of this gene. These regions conferred glucocorticoid inducibility by repressing the relatively high basal activity of the GS promoter in the absence of hormone. In the presence of hormone, activity was restored. This function may have implications for the tissue-specific induction of GS expression by glucocorticoids observed in lung and muscle during catabolic states.

METHODS

Reagents. Chemicals were purchased from Sigma (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). [32P]dCTP was purchased from NEN Life Science Products (Boston, MA). Dex (Sigma) was used as a 1 mM stock in absolute ethanol. Oligonucleotides were obtained from Genosys Biotechnologies (The Woodlands, TX). All tissue culture plasticware was obtained from Falcon (Becton Dickinson, Franklin Lakes, NJ). Tissue culture media, nutrients, serum, and antibiotics were obtained from GIBCO BRL (Life Technologies, Gaithersburg, MD).

Cell culture. Rat lung epithelial cells (L2 cells) and human prostate cancer cells (PC3 cells) were originally obtained from American Type Culture Collection (ATCC; Manassas, VA). L2...
and PC3 cells were grown in Ham's F-12K nutrient solution (Kaighn's modification) supplemented with 10% heat-inactivated fetal bovine serum, 4 mM additional glutamine, and 50 µg/ml of gentamicin. These cells were routinely passaged two times per week by trypsinizing and seeding at a 1:4 dilution in 15-cm-diameter tissue culture dishes. L2 cells were passaged the day before transfection with reporter plasmids, whereas PC3 cells were passaged 2 days before transfection, and the media were changed the day before.

Reporter plasmids. The reporter constructs containing up to 4.5 kb of the rat GS gene promoter region fused to the bacterial chloramphenicol acetyltransferase (CAT) coding sequence (here termed pGS117-CAT to pGS4.5-CAT) were kindly provided by Dr. Wouter H. Lamers (Department of Anatomy and Embryology, University of Amsterdam, Amsterdam, The Netherlands). These plasmids were constructed by inserting GS genomic fragments isolated from pGS3 into the multiple cloning site of the reporter plasmid pSS-CAT (51). To insert genomic fragments isolated from pGS3 into the dam, The Netherlands). These plasmids were constructed by inserting GS genomic fragments isolated from pGS3 into the pBluescript bacterial cloning vector (here termed pGS117-CAT to pGS4.5-CAT) were amplified with the oligonucleotide primer pair 5'-GGAGTCCGAGCTTTCTTTCTCTCTGCATAC-3' and 5'-GGGATCCACGTATTCTGAGCCTTACAACTTTACAG-3'. To construct pGS117i-CAT, the 569-bp Smal fragment within the first intron of pGS3 (+1,224 to +1,793 bp) 3' of the transcription start site) was inserted into the Smal site of pGS117-CAT. This fragment (corresponding to nucleotides 1107–1676 of European Molecular Biological Laboratories accession number X92074) contains a putative GRE located between nucleotides 1538 and 1552. This same Smal intron (iS) fragment was inserted in the forward and reverse (iSR) orientations at the Smal site of pT81-Luc to create pT81iBE-Luc and pT81iSR-Luc, respectively. To construct pT81iSH-Luc and pT81iHS-Luc, the unique HaeIII site (at nucleotide 1351) was used to divide the 569-bp Smal fragment into two smaller portions, a 244-bp Smal I-Hae III (iSH) fragment and a 325-bp Hae III-Sma I (iHS) fragment that includes the putative GRE, respectively. A BamHI-EcoR I fragment (bases 737–1938) containing 1.2 kb of the GS first intron and including the GRE was cloned into pBluescript SK + and then removed as a BamHI I-EcoR V (iBE) fragment and inserted between the BamHI I and Smal I sites of pT81-Luc to create pT81iBE-Luc. Thus this insert contains three bases derived from the pBluescript SK + vector.

Other plasmids used were pCMV-GR; the mouse mammary tumor virus (MMTV) promoter; kindly provided by Dr. Michael T. Crow, Laboratory of Cardiovascular Science, Gerontology Research Center, National Institute on Aging, National Institutes of Health, Bethesda, MD), pCDNA3-CAT [containing the cytomegalovirus (CMV) promoter; Invitrogen, Carlsbad, CA], pGL2-Control (containing the SV40 early promoter driving Luc expression; Promega), pCMV-Luc, pCDMB (Invitrogen), and pCDM8-GR (kindly provided by Dr. Ulupi J. Ihaa, Howard Hughes Medical Institute, Massachusetts General Hospital, Boston, MA). pCMV-Luc was constructed by replacing the Hind III-Bgl II fragment containing the SV40 promoter in pGL2-Control with a Hind III-Bgl II fragment from pCMV-SEAP (secreted placental alkaline phosphatase; Tropix, Bedford, MA). pCDM8-GR contains the cDNA for the rat GR inserted in the multiple cloning site of pCDM8 (U. J. Ihaa, personal communication).

Transfections and reporter assays. For transfection, subconfluent cells were harvested and washed with cold RPMI 1640 medium containing no supplements. Cells were suspended in cold RPMI 1640 medium at a density of 1 x 10^6 cells/ml. For transfections with CAT reporter plasmids, 20 µg of plasmid DNA and 200 µl of cell suspension (2 x 10^6 cells) were combined in an electroporation chamber with a 0.4-cm gap and subjected to a 330-µF discharge at 250 V with an electroporation device at a low-resistance setting (Cell-Porator, Gibco BRL, Life Technologies). The electroporated suspension was divided equally and plated in two 60-mm tissue culture dishes in complete Ham's F-12K medium (see Cell culture). Dex was added to obtain a 1 µM final concentration in one
Harvested by scraping. The samples were stored at after a 10-min incubation at room temperature, the cells were harvested by scraping. The samples were stored at -20°C until processed for reporter enzyme activity assays. For cotransfection and normalization of GS 5'-CAT reporter plasmid expression to Luc activity, 2 μg of pCMV-Luc plasmid DNA were combined with 8–20 μg of specific CAT-containing reporter plasmid DNAs (masses of CAT-containing plasmids added were adjusted to maintain a sixfold molar ratio of CAT-to Luc-containing plasmids based on 20 μg of pGS6.0-CAT). For cotransfection and normalization of GS promoter or intron fragment-CAT reporter plasmid expression to Luc activity, 5 μg of pGL2-Control plasmid DNA were combined with 20–23 μg of specific CAT-containing reporter plasmid DNAs (masses of CAT-containing plasmids added were adjusted to maintain a fivefold molar ratio of CAT-to Luc-containing plasmids based on 20 μg of pGS117-CAT). The cells were transfected as described above and plated in single 60-mm dishes with no drug addition. For cotransfection and normalization to CAT activity, 5 μg of pCDNA3-CAT were combined with 20 μg of specific Luc reporter plasmid DNAs, and cells were plated and treated with hormone as described above.

CAT activity was assayed according to the phase-extraction method of Kingston and Sheen (34) and Sheen and Seed (46). Briefly, the cell lysate was freeze-thawed three times, and 100 μl of the cell lysate were heat treated at 65°C for 15 min. An equal volume of reaction mixture containing 0.5 μCi/ml of [3H]-labeled chloramphenicol (NEN Life Science Products), 500 μM n-butyryl coenzyme A (Sigma), and 200 mM Tris, pH 7.5, was added. The mixture was incubated for 3 h at 37°C, extracted with 130 μl of a 1:2 mixture of xylene and 2,6,10,14-tetramethylpentadecane (Acros Organics, Pittsburgh, PA), and the radioactivity (in counts/min) in 100 μl of the upper organic phase was evaluated with a Topcount scintillation spectrophotometer (Packard Instruments, Meriden, CT).

Luc assays were performed with the Luc assay system (Promega) and a modified version of the manufacturer’s protocol. Briefly, after three cycles of freeze-thaw, the cell lysate was centrifuged at 12,000 g for 2 min at room temperature, 70 μl of cell lysate supernatant were placed in a well of a 96-well chemiluminescence plate (Liteplate, Packard Instruments), and an equal volume of Luc assay reagent was added. After mixing, the photon emission rate was measured with a Topcount scintillation spectrophotometer in single-photon count mode for 10 s/well.

Statistical analysis. Two-tailed Student’s t-test was utilized to estimate the significance of differences between groups. Significance was defined as a P value < 0.05.

RESULTS

Induction of GS 5’-reportor expression by Dex. A previous study in our laboratory (5) documented the induction of GS expression in rat L2 lung epithelial cells by the synthetic glucocorticoid hormone Dex. To identify portions of the GS promoter region necessary for glucocorticoid inducibility, the transient expression in L2 cells of a series of reporter plasmids containing CAT linked to progressively larger portions of the GS genomic clone (gGS3) was assayed (Fig. 1). The portions of the GS promoter utilized contained from 117 bp to ~6 kb of a contiguous sequence 5’ of the transcription start site and 59 bp 3’ of the transcription start site (to the Ear I restriction site). L2 cells were electroporated with these plasmids as well as with pMMTV-CAT and pCDNA3-CAT plasmids, plated identically into two dishes to which 1 µM Dex or ethanol carrier was added. CAT activity was assayed in the cellular lysates, and the multiple of induction was calculated as the ratio of radioactive counts in butyrylated chloramphenicol fractions (Fig. 2A). No apparent induction of expression by Dex was observed in any GS promoter-transfected cells except those containing pGS6.0-CAT, which includes the largest GS promoter fragment. It should be noted that inclusion of the ‘GRE-like’ element, which is ~406 bp upstream of the GS transcription site, did not bestow glucocorticoid inducibility on these plasmids. In contrast, inclusion of a 1.5-kb BamHI I fragment spanning 4.5–6.0 kb upstream of the GS transcription start site did confer glucocorticoid inducibility. Transient expression from pGS6.0-CAT was induced an average of fivefold by Dex. This induction was significantly greater than that of pGS117-CAT (P < 0.01) and all

Fig. 1. A schematic representation of glutamine synthetase (GS) 5’ promoter regions (negative nos. at left, location of 5’-end of each fragment in no. of bp relative to transcription (Txn) start site). Various GS reporter fragments included in gene constructs used for analysis of regulatory regions derived from genomic clone gGS3 and location of 1st intron region are shown. Positive nos. at bottom right, location of restriction enzyme cleavage site in no. of bp relative to Txn start site.
other GS reporter plasmids. This portion of the GS promoter region was then termed the GS-DIF. As a control, induction by Dex of expression from pMMTV-CAT and pCDNA3-CAT was assayed. Expression from pMMTV-CAT was induced an average of 61-fold, whereas average expression from pCDNA3-CAT was not induced by Dex. In these experiments, it was noted that basal CAT activity in pGS6.0-CAT-transfected cells was lower than that in cells transfected with other GS reporter fusion plasmids (data not shown). Rather than Dex causing an induction of CAT expression from pGS6.0-CAT to a relatively high level, this plasmid exhibited a relatively low basal level of CAT expression, which was induced to levels comparable to those of the other plasmids by Dex. It thus appeared that the GS promoter region lying within 4.5–6.0 kb upstream of the transcription start site acted as a repressor of transcription unless glucocorticoid hormone was present.

In addition to pGS6.0-CAT, pGS6.0R-CAT, which contained the 1.5-kb GS-DIFR, was constructed. These reporter plasmids, as well as pGS117-CAT, pGS4.5-CAT, and pMMTV-CAT, were electroporated into L2 cells, and CAT activities in lysates from cells in identically seeded plates cultured with or without Dex present in the medium were compared. CAT activity in lysates from pGS117-CAT- and pGS4.5-CAT-transfected cells was not significantly different and not significantly altered by culturing these cells in the presence and absence of Dex. CAT activity in lysates from pGS6.0-CAT- and pGS6.0R-CAT-transfected cells cultured in the absence of Dex was significantly (15.4- and 5.3-fold, respectively) lower than that from pGS4.5-CAT (P < 0.02 for each). GS expression from pGS6.0-CAT and pGS6.0R-CAT was induced by Dex (5.1- and 4.9-fold, respectively), whereas that from pGS4.5-CAT was not. CAT activity in lysates from pGS6.0-CAT- and pGS6.0R-CAT-transfected cells cultured in the presence of Dex was lower than that from pGS4.5-CAT-transfected cells in Dex, but these differences were not significant. Basal expression from pMMTV-CAT was nearly undetectable, and this plasmid was induced 47-fold by Dex.

To confirm that the basal expression from the GS promoter was inhibited by the region lying within 4.5–6.0 kb upstream from the transcription start site, L2 cells were cotransfected with equal molar amounts of CAT reporter plasmids along with 2 µg of pCMV-Luc and cultured in the absence of Dex. The ratio of CAT activity to Luc activity in the lysates was examined to control for variations in transfection efficiency as well as in cell harvesting and lysis (Fig. 2B). (It should be noted that normalization by Luc cotransfection was not performed for previous experiments because Luc activity in transfected cells exhibited an approximately twofold induction by Dex. This phenomenon was observed for Luc expression from viral promoters as well as from GS promoters and is believed to be due to a posttranscriptional effect on Luc expression.) When normalized for transfection efficiency, relative CAT expression from the plasmid containing only a small portion of GS promoter region, pGS117-CAT, was approximately one-half as active as that from pCDNA3-CAT, which contains the CMV long-terminal repeat (LTR). The slightly larger portion of the GS promoter in pGS368-CAT was significantly (2.7-fold) more active...
than the minimal GS promoter (P < 0.001) and at least as active as the CMV promoter. In contrast, basal expression from pMMTV-CAT was only 10% of that from pGS117-CAT. When greater portions of the GS promoter were included in pGS3.1-CAT and pGS4.1-CAT, normalized basal CAT activity was not significantly different from that with pGS117-CAT. In contrast, normalized CAT activity in cells transfected with pGS6.0-CAT and pGS6.0R-CAT was, on average, only 30% of that in pGS117-CAT-transfected and 40% of that in pGS4.5-CAT-transfected cells. Basal expression from pGS6.0-CAT and pGS6.0R-CAT was significantly lower than that of pGS117-CAT (P < 0.001 for each) and significantly lower than that of pGS4.5-CAT (P < 0.01 for each). Thus inclusion of the 1.5-kb GS-DIF in either orientation caused a reduction in transcription from the GS promoter in the absence of hormone.

Identification of glucocorticoid-responsive region within the GS-DIF.

As a first step to determine the sequences enabling the GS-DIF to act as a glucocorticoid-inducible repressor element, this 1.501-bp BamHI fragment was sequenced (Fig. 3). Examination of this
sequence revealed the presence of three GRE half-sites but no canonical GRE. These half-sites consist of the sequence TGTCT at bases 124–129, which corresponds to the high-affinity GRE consensus half-site (16), and two TGTCT sequences at bases 191–196 and 453–458, which correspond to the human metallothionein (hMT)-IIA GRE half-sites (31). In addition, a short interspersed element with 88–94% identity with members of the rodent interspersed DNA (ID) repeat element family (33) was discovered within the GS-DIF at bases 1340–1442.

To examine the role of these GRE half-sites, PCR amplification was used to obtain three subfragments of the GS-DIF of approximately equal sizes. These subfragments, termed GS-DIFA, GS-DIFB, and GS-DIFC, extended from bases 1 to 516, 506 to 961, and 945 to 1501, respectively (Fig. 4A). Thus DIFA contained all three GRE half-sites present in DIF and DIFC contained the ID element. DIF subfragments were inserted at the BamHI site of pGS4.5-CAT to construct pGS4.5DIFA-CAT, pGS4.5DIFB-CAT, and pGS4.5DIFC-CAT. Transfections into L2 cells demonstrated that DIFA alone conferred significant glucocorticoid inducibility to the 4.5-kb GS’ region (2.3-fold; P < 0.001 vs. pGS4.5-CAT; Fig. 4B).

Identification of a canonical GRE within the first intron of the GS gene. Recently, the portion of the first GS intron contained in a GS genomic clone was sequenced by Gaunitz et al. (25). This fragment contains a GRE between nucleotides 1538 and 1552, which corresponds to +1,656 to +1,670 bp 3’ of the GS transcription start site. The element contains the sequence AGAACATATTGTTCC (Fig. 5), which in reverse orientation closely resembles a classic canonical GRE (GGTACANNNTGTTCT) (12). In forward orientation, the GS intron GRE sequence also closely resembles the hormone response element consensus (AGAACANNNTGTTCT) for glucocorticoid, mineralcorticoid, progesterone, and androgen receptors (23).

Effect of GS-DIF and the GRE-containing fragment of the first GS intron on the activity of a minimal GS promoter. The reporter gene construct containing a relatively small GS promoter fragment, pGS117-CAT, was found to possess considerable transcriptional activity (Fig. 2B). This construct was therefore used as a basis to examine and compare the effects of the GS-DIF and the GS intron fragment on the basal and Dex-induced activities of a “minimal GS promoter.” The GS-DIF was inserted into pGS117-CAT in forward and reverse orientations to test its effects. pGS117DIF-CAT and pGS117DIFR-CAT are therefore equivalent to pGS6.0-CAT and pGS6.0R-CAT, respectively, with the BamHI–ApaI fragment between –4,500 and –117 bp removed (see Fig. 1). These plasmids, along with pGS117-CAT and pMMTV-CAT, were electroporated into L2 cells, and CAT activity was compared in lysates from cells that were cultured in identically seeded plates with and without Dex present in the medium for 72 h (Table 1). Expression from pGS117-CAT exhibited no Dex induction. Inclusion of the GS-DIF in the forward and reverse orientations conferred significant
Table 1. Effect of GS-DIF on minimal GS promoter

<table>
<thead>
<tr>
<th>Plasmid Construct</th>
<th>Multiple of Induction by Dex</th>
<th>n</th>
<th>Relative Basal Expression</th>
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<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGS117-CAT</td>
<td>0.97 ± 0.14</td>
<td>6</td>
<td>1.0</td>
</tr>
<tr>
<td>pGS117DIF-CAT</td>
<td>2.5 ± 0.4†</td>
<td>6</td>
<td>0.46 ± 0.28†</td>
</tr>
<tr>
<td>pGS117DFR-CAT</td>
<td>3.7 ± 0.7†</td>
<td>6</td>
<td>0.60 ± 0.19†</td>
</tr>
<tr>
<td>pMMTV-CAT</td>
<td>46 ± 36*</td>
<td>6</td>
<td>0.12 ± 0.14†</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
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<tr>
<td>pGS117-CAT</td>
<td>1.3 ± 0.7</td>
<td>6</td>
<td>1.0</td>
</tr>
<tr>
<td>pGS117-CAT</td>
<td>3.9 ± 1.4†</td>
<td>6</td>
<td>0.29 ± 0.12†</td>
</tr>
<tr>
<td>pGSMMTV-CAT</td>
<td>26 ± 14†</td>
<td>6</td>
<td>0.11 ± 0.08†</td>
</tr>
<tr>
<td>pCDNA3-CAT</td>
<td>ND</td>
<td>1</td>
<td>0.1 ± 0.5</td>
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Values are means ± SD except average = difference for pCDNA3-chloramphenicol transferase (CAT); n, no. of transfections performed. CAT activity was determined as described in METHODS. Multiples of induction were calculated as ratio of CAT activity in lysates from cells cultured in presence of dexamethasone (Dex) to that in cells cultured in presence of ethanol carrier alone. To determine relative basal expression levels, L2 cells were cotransfected with each of the glutamine synthetase (GS) Dex-inducible fragment (DIF) reporter constructs indicated along with pCMV-luciferase at a 6:1 molar ratio (experiment 1) or with indicated intron (i) fragment-containing plasmids along with pGL2-Control at a 5:1 molar ratio (experiment 2). CAT and luciferase activities were determined, and CAT activity was normalized as described in METHODS. DIFA, DIFA2 in reverse orientation; MMTV, mouse mammary tumor virus; ND, not done. Significantly different from pGS117-CAT-transfected cells: *P < 0.05; †P < 0.01; ‡P < 0.001.

Dex inducibility on the basal GS promoter, with inductions for pGS117DIF-CAT and pGS117DFR-CAT of 2.5- and 3.7-fold, respectively (P < 0.01 for each). MMTV-CAT exhibited a mean of 46-fold Dex inducibility in these transfections.

Similar to when the GS-DIF was included in the 4.5-kb GS promoter construct (Fig. 2B), GS-DIF caused a marked depression in basal expression from the minimal GS promoter in the absence of Dex (data not shown). To confirm this observation, L2 cells were then cotransfected with equal molar amounts of these reporter plasmids along with 2 µg of pCMV-Luc and cultured in the absence of Dex (Table 1). The basal activity exhibited by pGS117DIF-CAT and pGS117DFR-CAT was 46 and 60%, respectively, of that of pGS117-CAT (P < 0.01 for each). Thus GS-DIF conferred Dex inducibility and repressed the basal activity of the minimal GS promoter. Basal expression from pMMTV-CAT was 12% of that of pGS117-CAT.

To test the capacity of the GS intron fragment on the basal and Dex-induced activities of the minimal GS promoter, the 569-bp Sma I fragment from pGS3, which contains the GRE, was fused to the GS basal promoter in pGS117-CAT to form pGS117S-CAT. The induction of expression from this reporter plasmid was compared with that of pGS117-CAT and pMMTV-CAT after transfection into L2 cells (Table 1). Expression from pGS117-CAT was not significantly increased by Dex. Inclusion of the GS intron fragment conferred significant Dex inducibility on the basal GS promoter of 3.9-fold (P < 0.01). MMTV-CAT exhibited a mean of 46-fold Dex inducibility in these transfections.

Similar to the upstream GS-DIF, the intron fragment seemed to reduce the basal level of expression in the absence of Dex. CAT activity in pGS117-CAT-transfected cells was relatively high in both the presence and absence of Dex. In the absence of Dex, average expression from pGS117S-CAT was significantly lower than that from pGS117-CAT (2.5-fold; P < 0.01), whereas Dex induced expression from pGS117S-CAT to 33% higher than the level of pGS117-CAT (not significant). To confirm the ability of the intron fragment to repress expression from the minimal GS promoter, L2 cells were cotransfected with equal molar amounts of pGS117-CAT, pGS117S-CAT, pMMTV-CAT, or pCDNA3-CAT along with pGL2-Control (SV40-Luc) in a 5:1 molar ratio and plated in the absence of Dex, and the ratio of CAT activity to Luc activity was examined (Table 1). In this experiment, CAT expression from pGS117-CAT was approximately as active as that from pCDNA3-CAT. Basal expression from pGS117S-CAT was significantly reduced to only 29% of that from pGS117-CAT (P < 0.01), demonstrating that inclusion of the 569-bp Sma I fragment from the GS intron caused a considerable repression in basal transcription. Basal expression from pMMTV-CAT was 11% of that of pGS117-CAT in this experiment.

GS-DIF and the GS Intron contain regions that act as Dex-responsive enhancers. The ability of smaller fragments of the GS-DIF and the GS intron to confer Dex-dependent enhancement of transcription from a heterologous promoter was tested. The minimal HSVtk promoter was chosen because it is often used to test enhancer function. This minimal promoter also proved to be much more sensitive to the influence of small GS fragments than the minimal GS promoter. PCR amplification was used to obtain further subdivisions of DIFA. These fragments, termed DIFA1 and DIFA2, included bases 1–255 and 221–516, respectively (see Fig. 5). DIFA1 included a consensus DIF element as well as one MT-IIA GRE half-site, and DIFA2 included one MT-IIA GRE half-site. These GS-DIFs were inserted into reporter plasmids containing a cDNA-encoding firefly Luc fused to an 81-bp portion of the HSVtk promoter pT81-Luc (39). These heterologous reporter plasmids, as well as pT81-Luc, were electroporated into L2 cells along with pCDNA3-CAT, and normalized Luc activity in lysates from cells in identically seeded plates cultured with and without Dex present in the medium was compared (Fig. 6A). Only pT81DIFA-Luc and pT81DIFA1-Luc exhibited significantly greater Dex inductions than pT81-Luc (P < 0.02 for each). Whereas expression from pT81-Luc was induced 2.1-fold by Dex, expression from pT81DIFA-Luc and pT81DIFA1-Luc was induced ~8.7- and 8.8-fold, respectively. Thus a glucocorticoid-responsive region seems to be located in a 225-bp segment lying ~6 kb 5′ of the transcription start of GS.

In general, the GS-DIFs did not act as repressors when fused to the HSVtk minimal promoter. Comparison with pT81-Luc suggested that basal Luc activity in the absence of Dex was enhanced by fusion of DIFA, DIFB, DIFA1, and DIFA2 (data not shown). DIFB and DIFA2 actually enhanced expression similarly with and without hormone, whereas enhancement by DIFA...
and DIFA1 was markedly increased in the presence of Dex. Therefore, DIFA and DIFA1, like the intact GS-DIF, acted as glucocorticoid-dependent enhancer elements. In contrast, DIFC, which contained the ID element, markedly depressed expression from the HSVtk minimal promoter in both the presence and absence of Dex (data not shown).

To further examine the role of the GRE in Dex inducibility conferred by the GS intron, the 569-bp GRE-containing SmaI intron fragment was inserted in the forward and reverse orientations into the SmaI site of pT81-Luc to create the plasmids pT81iS-Luc and pT81iSR-Luc. In addition, an internal HaeIII site was utilized to create pT81iSH-Luc and pT81iHS-Luc containing the proximal and distal portions of the SmaI fragment. When cotransfected into L2 cells along with pCDNA3-CAT and normalized to CAT activity, Luc expression from all these reporter plasmids was induced to some degree by Dex, including pT81-Luc that, on average, was induced 5.0-fold in these experiments (Fig. 6B). However, expression from pT81iS-Luc was induced 22-fold and pT81iSR-Luc was induced 8.2-fold by Dex. Expression from pT81iHS-Luc (which includes the GRE-containing portion of the intron fragment) was induced 30-fold by Dex, whereas pT81iSH-Luc exhibited 4.6-fold Dex inducibility or no more than pT81-Luc itself. Thus inclusion of intron fragments increased Dex inducibility, but this induction seemed to be orientation dependent such that only the inductions of pT81iS-Luc and pT81iHS-Luc were significantly greater than that of pT81-Luc (P < 0.04 and P < 0.01, respectively). Therefore, the glucocorticoid inducibility of the GS intron fragment seemed to be localized to the 325-bp HaeIII-SmaI fragment containing a nearly canonical GRE.

Unlike when the GS intron fragment was fused to the minimal GS promoter, intron fragments did not act as a repressor of the HSVtk promoter. Rather, basal expression was enhanced by fusion to the GS intron SmaI fragment or portions thereof, and enhancement by the iS and iSH fragments was increased in the presence of Dex (data not shown). It should be noted that in comparison with CMV promoter, it seemed that the minimal HSVtk promoter was much less transcriptionally active than the minimal GS promoter (data not shown).

Dependence on the GR for induction of GS reporter plasmid expression by Dex. To test whether the presence of GR is necessary for induction of GS reporter gene expression by Dex, a human prostate carcinoma cell line with undetectable levels of GR expression was utilized (27). PC3 cells were cotransfected with GS reporter plasmids or pMMTV-CAT along with an expression plasmid containing a cDNA encoding the rat GR (pCMD8-GR) or a control plasmid containing no cDNA insert (pCMD8). pCMD8-GR or control was cotransfected into PC3 cells along with pGS117-CAT, pGS117iS-CAT, pGS4.5-CAT, pGS6.0-CAT, pGS6.0R-CAT, or pMMTV-CAT; the effect of Dex on CAT activity was assayed (data not shown). None of these plasmids was responsive to Dex in the absence of exogenous GR

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**Fig. 6. Localization of Dex-responsive enhancers in GS-DIF and GS intron.** A: different regions of GS-DIF were PCR amplified and cloned upstream of herpes simplex virus thymidine kinase (HSVtk) promoter plasmid pT81-Luc expressing Luc. L2 cells were transfected with plasmids as indicated along with pCDNA3-CAT and then cultured with either Dex or ethanol for 72 h. Luc and CAT activities were determined as described in METHODS. Luc activity was normalized to CAT activity, and multiple of induction was calculated as ratio of normalized Luc activity in presence of Dex to that with ethanol carrier alone. Values are means ± SD for 3 transfections. *P < 0.02 relative to pT81-Luc-transfected cells. B: SmaI region of intron segment was further subdivided into 2 regions and cloned proximal to HSVtk promoter in pT81-Luc plasmid. pT81iHS-Luc contained the consensus GRE. L2 cells were transfected with Luc plasmid as indicated along with pCDNA3-CAT and treated with either Dex or ethanol carrier. Luc and CAT activities were determined as described in METHODS. Luc activity was normalized to CAT activity, and multiple of induction was calculated as ratio of normalized Luc activity in presence of Dex to that with ethanol carrier alone. Values are means ± SD for 3 transfections. *P < 0.04 and @P < 0.01 relative to pT81-Luc-transfected cells.
expression. When PC3 cells were cotransfected with GR, Dex caused a significant increase in CAT expression from all of these reporter plasmids, including pGS117-CAT, which, on average, were induced 2.7-fold \((P < 0.01)\). Therefore, the presence of an active glucocorticoid response unit seemed to cause a nonspecific induction of CAT activity. Inclusion of the GRE-containing intron fragment in pGS117iS-CAT increased Dex induction to 5.2-fold. Dex induction of pGS4.5-CAT expression was 4.1-fold in the presence of exogenous GR expression. Average expression of pGS6.0-CAT and pGS6.0R-CAT, which include the 1.5-kb GS-DIF in opposite orientations, was induced 10.3- and 11.0-fold, respectively, by Dex when GR was coexpressed. In comparison, pMMTV-CAT expression was induced 7.8-fold by Dex when PC3 cells were cotransfected with pCDM8-GR. Therefore, Dex induction was enhanced by inclusion of the GS intron fragment or the GS-DIF, and this effect was dependent on the presence of the GR. However, none of the enhanced inductions was highly significant relative to pGS117-CAT in the presence of GR and Dex \((0.04 < P < 0.3)\). Although CAT activity was not normalized to an internal control, it was apparent that basal expression in the absence of Dex was repressed by inclusion of GS-DIF and the intron fragment and that this repression was relieved by Dex only when GR was cotransfected (data not shown).

A BamH I-EcoRI I fragment containing 1.2 kb of the GS first intron (bases 737–1938), including the GRE, was inserted between the BamH I and Sma I sites of pT81-Luc to create pT81IBE-Luc. Additionally, the 1.5-kb BamH I GS-DIF was inserted in both the forward and reverse orientations to create pT81DIF-Luc and pT81DIFR-Luc, respectively. PC3 cells were cotransfected with these heterologous reporter plasmids along with pCMV8-G or pCMV8, and the effect of Dex on Luc activity was assayed (Fig. 7). For none of the constructs was Luc expression responsive to Dex in the absence of exogenous GR expression. When PC3 cells were cotransfected with GR, Dex caused an increase in Luc expression from all of these reporter plasmids. However, expression from pT81-Luc was induced only 1.8-fold. Fusion of the GRE-containing intron fragment to the HSVtk promoter in pT81i-Luc increased Dex induction 4.7-fold. Dex induction of pT81DIF-Luc and pT81DIFR-Luc expression was 5.6- and 19.4-fold, respectively, in the presence of exogenous GR expression. Thus both the GS intron fragment and GS-DIF were able to confer significantly increased glucocorticoid inducibility on expression from the minimal HSVtk promoter in a GR-dependent fashion \((P < 0.01\) for each). Inclusion of the GS-DIF and the intron fragments did not cause a significant reduction in basal expression from the minimal HSVtk promoter in PC3 cells but instead enhanced expression, particularly in the presence of Dex and GR (data not shown).

**DISCUSSION**

During catabolic states, the lung increases its output of glutamine \((7, 8, 48)\). Concurrent with this response is an increase in GS activity. In the rat lung, GS mRNA level is normally low compared with many other tissues but is increased severalfold during catabolic states \((3, 6, 7, 10, 11)\). Rat GS expression is markedly induced by glucocorticoids both in the whole lung after Dex injection and in cultured rat lung cells, and adrenal-derived hormones play a large role in the induction of GS gene expression in the lungs of endotoxemic rats \((1, 4, 5)\). Although glucocorticoid hormones seemingly play a large role in the control of GS gene expression during catabolic states, the genetic elements responsible for this regulation have not been identified.

Reporter plasmids containing portions of rat GS genomic sequences both upstream of the transcription start and within the first intron were transfected into rat L2 cells as well as into human PC3 cells to determine the sequences necessary for the induction of GS expression by activated GR. L2 cells are a rat epithelial line that was originally derived through clonal isolation from a rat alveolar type II cell primary culture \((30)\). L2 cells exhibited phosphatidate phosphohydrolase-specific activity and an immunohistochemical reactivity pattern similar to alveolar type II cells \((19)\). However, the ATCC reports that in the later passage cultures provided, lamellar inclusions are not apparent and the rates of phosphatidylcholine synthesis are comparable to those exhibited by fibroblasts. Thus L2 cells cannot be considered truly representative of alveolar type II cells. Nevertheless, induction of GS mRNA and protein expression in L2 cells by Dex was previously found to be a direct GR-dependent response...
HORMONE-SENSITIVE REPRESSOR ELEMENTS IN RAT GS GENE

In chicken retina explants, mouse adipocytes, and rat myoblasts and glial cells, glucocorticoids have been shown to cause an increase in GS transcription rate (14, 22, 35, 42). Glucocorticoid induction of GS gene expression in chicken retina has been conclusively demonstrated to be due to GRE-dependent activation of transcription, and the chicken GS gene is known to contain eight GREs within 2.6 kb 5' of the transcription start site (52). In contrast, sequencing of 2.5 kb of the rat GS promoter revealed only a single GRE-like sequence element located 406 bp 5' of the transcription start (21, 38). However, this sequence (GGTAAAA-TTCTCT) bears little resemblance to a canonical GRE or to GRE half-sites. Fahrenholtz et al. (21) found that the region containing this GRE-like sequence did not influence Dex induction of GS promoter constructs in rat hepatoma cells. The present study found no evidence that this GRE-like sequence is sufficient for glucocorticoid induction of transcription in L2 cells. Instead, two regions of the GS gene located nearly 6 kb 5' of the transcription start and within the first intron were found to act as glucocorticoid-inducible elements. More precisely, both of these regions repressed basal expression from the GS promoter, and this repression was relieved in the presence of glucocorticoid.

Analysis of contiguous fragments of the GS promoter region from +59 bases up to −6.0 kb from the transcription start site revealed a lack of glucocorticoid inducibility until inclusion of a 1.5-kb fragment spanning −4.5 to −6.0 kb. This fragment, termed GS-DIF, acted as a GRE when fused directly to the GS promoter as well as to a heterologous promoter. GS-DIF contains three GRE half-sites in the distal portion of this fragment, including a high-affinity consensus half-site (TGTCTT) (16) and two hMT-IIA half-sites (TGTCTT) (31, 32). The high-affinity GRE half-site is present in a number of GREs, including MMTV-I, MMTV-IIA (43), tyrosine aminotransferase (28), rat plasminogen activator inhibitor-1 (15), rat serine dehydrogenase (49), and rabbit uteroglobin (29). The hMT-IIA half-site is present in a number of genes, including human growth hormone (47) and rat chromogranin A (44), and this element also functions as an androgen response element (17). Dexamethasone (Dex) inducibility was retained in a 255-bp GS-DIF fragment containing the two most distal half-sites, one consensus and one hMT-IIA. Further mutational analysis is required to determine the role of individual sites in the glucocorticoid responsiveness of this fragment.

A portion of the first GS intron was studied after examination of the published sequence revealed the presence of a nearly canonical GRE, with the sequence AGAACATATTGTCTCC. The intron portion containing this GRE did confer Dex inducibility to both the GS basal promoter and the heterologous HSVtk promoter in a GR-dependent fashion. The glucocorticoid inducibility of the GS intron fragment was localized to the 325-base HaeIII-SmaI fragment containing this GRE. When inverted, all but the 13th residue (underlined) of the GS intron sequence matches the GRE consensus sequence (GGTACANNNTGTTCTT) (12). Analysis of MMTV GRE mutations suggested that substitution at the third position of the canonical GRE does not appreciably impair activity (40). In fact, the half-site (TGTCTC) is also present in all three of the human elastin promoter GREs (18). Further mutational analysis is required to confirm the role of this hormone response element in the hormonal induction of this fragment.

The smallest GS reporter fragment (bases −117 to +59) exhibited relatively high transcriptional activity in L2 cells that was comparable to that of the CMV promoter. Larger GS promoter fragments retained relatively high activity, with the exception of the full 6.0-kb fragment. Inclusion of the 1.5-kb GS-DIF caused a decline in basal activity in the absence of hormone; activity was restored in the presence of Dex. Fusion of the GS-DIF to the minimal GS promoter region (bases −117 to +59) repressed basal activity and conferred Dex inducibility. Similarly, fusion of a GRE-containing GS intron fragment to the minimal GS promoter resulted in a reduced basal activity, which was restored in the presence of Dex. Thus the glucocorticoid-responsive GS fragments seemed to act not so much as enhancers but rather as hormone-sensitive repressors. Similarly, the GREs of the MMTV LTR, the ovine β2-adrenergic-receptor promoter, and the progesterone-responsive region of the chicken ovalbumin promoter all repress transcription from their homologous promoters (20, 24, 50). In addition, the glucocorticoid-responsive region of human cytochrome P450B4 suppressed expression from a 156-bp portion of the HSVtk promoter (45). In the case of the MMTV LTR, repression is caused by nucleosome positioning about the GRE (13). Binding of the GR releases this repression by stimulating nucleosome disruption by the SWI/SNF complex, thus allowing additional transcription factors to bind (41). It remains to be determined whether glucocorticoid regulation of GS operates in an analogous fashion.

GS-DIF and its GRE-containing fragments did not repress basal expression from the HSVtk minimal promoter. Rather, GS-DIF, DIF-A, and DIF-A1 all acted as glucocorticoid-dependent enhancer elements in this context (data not shown). DIF-B and DIF-A2 enhanced expression from the HSVtk promoter similarly with and without hormone. Only DIF-C, which contained the ID element, markedly depressed expression from the HSVtk minimal promoter both in the presence and absence of Dex (data not shown). The significance of this repression is not known. Similarly, GS intron fragments did not repress basal expression of the minimal HSVtk promoter but rather enhanced it in the presence of Dex. In contrast to the GS basal promoter, this 81-bp portion of the HSVtk promoter exhibited relatively low basal activity. Thus hormone-responsive GS fragments exhibited the characteristics of steroid-
relied repressors when coupled to the relatively strong GS promoter and of steroid-dependent enhancers when coupled to a relatively weak HSVtk promoter.

The glucocorticoid responsiveness of both the GS promoter and intron elements was dependent on the expression of GR. In PC3 cells, reporter plasmids containing these elements showed no induction by Dex unless exogenous GR was cotransfected (Fig. 7 and data not shown). Interestingly, expression of exogenous GR seemed to cause a nonspecific induction of all reporter plasmids by Dex. This was not unique to the HSVtk promoter-linked Luc because cells transfected with SV40 promoter- and CMV promoter-driven Luc also exhibited nonspecific induction of expression by Dex (data not shown). Nonetheless, inclusion of GS fragments into reporter plasmids caused a heightened induction of expression by Dex in GR-expressing cells. Preliminary results have demonstrated that the GS intron-residing GRE and the region of the GS-DIF containing GRE half-sites are able to bind purified GR (Chandrasekhar, unpublished data). Footprinting studies are required to confirm GR binding to the individual half-sites and to determine the actual bases being contacted by the GR.

The rat genomic clone gGS3 contains two glucocorticoid-responsive regions, one nearly 6 kb upstream from the transcription start site and another within the first intron of the gene. A number of genes are known to contain GREs within their first intron. There are also a number of genes that contain functional GREs > 5 kb upstream from the transcription start sites. The responsiveness of GS reporter plasmids to various hormones was recently compared with that of endogenous GS expression (Chandrasekhar and Abcouwer, unpublished observations). GS-DIF-containing plasmids were inducible only by glucocorticoid hormones, whereas the intron fragment-containing plasmid was comparably induced by progesterone and estradiol as well as by glucocorticoids. Although GS mRNA levels were much more responsive to glucocorticoid induction, GS expression was also induced by progesterone and estradiol. This intermediate response might be expected if the inducibility of endogenous GS is determined by the cooperative influences of both the far-upstream and the intron elements. Furthermore, it is conceivable that GS expression is also influenced by additional distal GREs located outside the genomic clone gGS3.

A recent report by Hadden et al. (26) identified a region responsible for GS induction during adipocyte differentiation located in the first intron of the mouse GS gene between bases +1600 and +1900. This enhancer region was activated during differentiation of 3T3-L1 cells treated with dexamethasone, methylisobutylxanthine, and insulin. However, this region was not responsive to glucocorticoid alone, and no GRE was identified. These authors also demonstrated that only a composite fusion gene containing the mouse GS intron fragment together with an upstream fragment (+3 kb) accurately mimicked induction of endogenous GS expression during adipocyte differentiation. Similarly, Gaunitz et al. (25) have reported transcriptional enhancers that regulate rat GS expression in hepatocellular cultures lying within the first intron and 6.5–8 kb upstream from the transcription start site.

Thus it may be speculated that the glucocorticoid regulation of rat GS expression is, at least in part, regulated by transcriptional repression in the absence of hormone caused by two separate elements within this gene. It has yet to be determined whether this repression is tissue specific, thus leading to the preferential glucocorticoid response of GS expression in lung and muscle tissue. The basal expression level of GS in both rat lung and muscle is low relative to several other organs (1). The relative glucocorticoid inducibility of GS in lung and muscle may stem from relief of transcriptional repression, which does not operate in tissues with a higher basal expression. In turn, glucocorticoid induction of GS expression may support the ability of lung and muscle to increase glutamine synthesis in catabolic states.

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