Glucocorticoid receptors are ligand-dependent transcription factors that are subject to down-regulation by their cognate ligand; however, the mechanisms mediating this physiological response are not completely understood. Since analysis of the human glucocorticoid receptor (hGR) cDNA sequence revealed the presence of sequences with homology to both positive and negative glucocorticoid regulatory elements, we have examined the potential of hGR to bind to the hGR cDNA by Southwestern blot analysis. The data revealed that glucocorticoid receptors exhibited specific binding to their own cDNA. To determine whether this binding was of functional significance in the down-regulation of glucocorticoid receptors, we analyzed the effect of glucocorticoids on hGR protein levels from COS 1 cells transfected with an hGR cDNA expression vector. These transfected cells produced intact hGR that were capable of ligand-dependent regulation of a co-transfected glucocorticoid-responsive reporter gene. Glucocorticoid treatment of hGR-transfected cells resulted in down-regulation of hGR (assayed by both glucocorticoid binding capacity and hGR protein levels) within 24 h of steroid administration. To determine if the glucocorticoid-induced down-regulation of transfected hGR was compatible with effects at the levels of receptor gene expression and RNA stability, we examined hGR mRNA steady state levels. Reductions from 2- to 6-fold were observed in hGR mRNA levels following glucocorticoid treatment of transfected COS 1 cells. This down-regulation of transfected hGR mRNA could not be attributed to either the Rous sarcoma virus promoter, which drives hGR expression, or to other sequences present in the vector plasmid since transcription of a related plasmid containing a chloramphenicol acetyltransferase gene in place of the hGR cDNA was not regulated by glucocorticoids. Down-regulation of hGR mRNA by glucocorticoids in transfected cells occurred in a time- and dose-dependent manner that is consistent with a glucocorticoid receptor-mediated process. Glucocorticoid-induced down-regulation of hGR mRNA steady state levels was not observed in COS 1 cells transfected with cDNAs encoding mutant hGR (defective in either steroid or DNA binding), which indicates that functional steroid and DNA binding domains of the expressed hGR were required for down-regulation. Interestingly, treatment of transfected COS 1 cells with the glucocorticoid antagonist RU486 also resulted in down-regulation of transfected hGR mRNA. Deletion analysis revealed that the region of the hGR cDNA that was responsible in part for the observed down-regulation in response to glucocorticoid was contained within a 1-kilobase restriction fragment (from base pair +527 to +1526). Together these studies provide evidence that the hGR cDNA contains regulatory signals sufficient for down-regulation of glucocorticoid receptors.

Both positive and negative modulation of gene transcription in response to glucocorticoids has been well established (for review see Refs. 1-3). The action of glucocorticoid hormones is mediated through specific high affinity binding to glucocorticoid receptors (GR). These proteins, together with other steroid receptors, belong to a family of ligand-dependent transcription factors (4). Following steroid binding, the receptor hormone complex interacts with DNA and ultimately results in alteration of the transcription of certain genes. Although the mechanism of steroid hormone action has not been completely elucidated, it is known that the GR complex interacts with specific target DNA sequences called glucocorticoid regulatory elements (GREs) that are associated with genes whose transcription is elevated in response to hormone. Much less is known about how GR negatively regulates gene expression; however, sequences that may serve as negative GREs (nGREs) have been recently identified for a few genes whose transcription is inhibited by glucocorticoids (5).

The biologic effects of glucocorticoids are dependent on the presence of functional receptors (6). One determinant of cellular responsiveness to steroid treatment is the concentration of GR (7, 8). Thus, to understand the mechanism of steroid hormone action it is of particular importance to understand how cellular GR levels are regulated. Glucocorticoids and other steroid hormones down-regulate the levels of their cognate receptors in a number of target tissues and in many different cell lines (9-11). Receptor down-regulation has been documented previously for certain peptide hormone receptors and receptors for neurotransmitters, and this phenomenon

* This work was supported in part by National Institutes of Health Grant DK32460. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by National Institutes of Health Postdoctoral Fellowship F32-GM10866 and Training Grant T32 CA00156 (Lineberger Cancer Research Center). To whom correspondence should be addressed: Dept. of Physiology, 460 Med. Sci. Res. Bldg., C.B. 7545, University of North Carolina, Chapel Hill, NC 27599-7545.
has been correlated with "desensitization" to prolonged hormone treatment (reviewed in Ref. 12). Autoregulation of GR was initially defined as a decrease in steroid binding capacity following glucocorticoid treatment; more recently, reduced steady state levels of GR mRNA (13, 14) and GR protein (15, 16) have been demonstrated. Although the mechanism for GR down-regulation occurs, at least in part, at the level of gene transcription (17), several groups have also examined the influence of ligand on GR turnover. McIntyre and Samuels (18) used dense amino acid labeling to show that GR half-life (as measured by steroid binding) in a pituitary tumor cell line is shortened following glucocorticoid treatment. Glucocorticoid-induced changes in protein turnover have also been documented using protein synthesis inhibitors in a rat liver cell line (15). In contrast, when a pulse-chase labeling procedure and quantitation by antibody binding were employed to study GR protein half-life in hormone-treated S49 mouse lymphoma cells, no changes in receptor half-life were seen (19). Such discrepancies may reflect cellular variations in receptor recycling mechanisms (20) or post-translational modifications of receptor that are essential for ligand binding but not perhaps for recognition of receptor by antibodies.

Whether GR mediates down-regulation through binding to GRE-like elements that are associated with the GR gene, as shown previously for positively regulated genes, is not known. Okret et al. (13) reported that certain sequences in the 3'-nontranslated region of a rat GR cDNA fragment are protected from DNase I digestion by partially purified GR; however, it is unclear whether these binding sites are functionally involved in down-regulation of GR mRNA and protein.

The results presented here indicate that the hGR cDNA encodes sufficient information to display glucocorticoid-induced down-regulation of hGR mRNA and protein levels when it is transfected into COS I cells. Both the glucocorticoid agonist, dexamethasone, and antagonist, RU486, elicited the down-regulatory response in transfected cells. We did not observe down-regulation in the absence of functional DNA or steroid binding domains of the receptor suggesting that down-regulation is receptor-mediated. Deletion of 1 kb of hGR cDNA-coding sequences resulted in a truncated hGR mRNA that was impaired in its ability to undergo down-regulation perhaps due to the removal of signals important in hGR regulation. Taken together the data presented here suggest that the human glucocorticoid receptor contains intragenic regulatory sequences.

MATERIALS AND METHODS

[3H]Dexamethasone mesylate (37.5 Ci/mmol), [14C]Chloramphenicol, and ENHANCE were purchased from Du Pont-New England Nuclear. [α-32P]UTP (600 Ci/mmol), α-[32P]dCTP (3000 Ci/mmol), and [32P]-5′-paps-oligo(dT)-cellulose were supplied by New England Nuclear (Boston, MA). Dextran sulfate was from Pharmacia (Piscataway, NJ). [3H]Chloramphenicol was kindly provided by Dr. R. Daradzik, Roussel UCLAF (Romainville, France). Restriction enzymes and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, New England Biolabs (Beverly, MA), and Promega Corp. (Madison, WI). Nitrocellulose B85 was from Schleicher & Schuell. Pulsed amino acid labeled standards were purchased from Bethesda Research Laboratories. Cell Culture and Transfection—COS I cells (African green monkey kidney cells) (21) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 9 mg/ml glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin and supplemented with 2 mM L-glutamine and 10% (v/v) of a mixture (1:1) of fetal calf serum/cell culture medium. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2. Cell number was determined using a Zf Coulter Counter (Coulter Electronics, Hialeah, FL). Cells were transfected by the DEAE-dextran method of Sompayrac and Danne (22) as modified by Gorman (23). Cells were refed with supplemented DMEM after a 3-h incubation with DNA.

Recombinant Plasmids—The hGR expression vector, pRSShGR (24), consists of an ~3.0-kb hGR cDNA driven by the Rous sarcoma virus long terminal repeat promoter (RSV-LTR) and contains the simian virus 40 (SV40) origin of replication and polyadenylation sites. Plasmids J422 and J582 (24) are in-frame BamHI linker insertion mutants of pRSshGR that are incapable of DNA and steroid binding, respectively. The number designation of each mutant refers to the hGR amino acid position after which the linker was inserted (24). All hGR plasmids were generously provided by Drs. Stanley Hollenberg and Ron Evans (Salk Institute, San Diego, CA). Plasmid pRSVCAT/ori, which contains the SV40 origin of replication, was constructed by ligating the ~350-bp pRSshGR Ndel fragment to pRSV CAT/ori (kindly provided by Dr. Robert E. Gutter University of California, San Francisco, CA) that had been linearized with Ndel and phosphorylated. The identity of the clone was verified by restriction enzyme mapping and hybridization. The resulting recombinant plasmid pRSV CAT/ori is therefore identical to pRSshGR except that the hGR cDNA has been replaced by the chloramphenicol acetyltransferase (CAT) gene. The hGR deletion mutant, ΔAcc-Cla hGR, was created by removing the 1000-bp AccI to Clal fragment (+527 to +1526) from the hGR cDNA and religating the plasmid. ΔAcc-Cla hGR encodes an ~2.3-kb transcript that is not in-frame.

RNA Isolation and Northern Blot Analysis—RNA was isolated from transfected cells by the rapid cell lysis method of Radley et al. (25) by homogenization in guanidine isothiocyanate and centrifugation through a cesium chloride cushion (26). When indicated, poly(A)+-enriched RNA was obtained by binding and eluting RNA from oligo(dT)-cellulose (Collaborative Research, Lexington, MA). RNA was denatured using glyoxal and dimethyl sulfoxide, separated by electrophoresis through 1% agarose gels, and transferred to nitrocellulose filters as described by Maniatis et al. (27). Filters were hybridized by standard methods using randomly primed 32P-labeled hGR cDNA and chick β-actin cDNA (~1 × 106 cpm/ml hybridization fluid) or with 32P-labeled cDNA probes (~3−5 × 106 cpm/ml). The dual promoter vector pT7/T3-18 (Bethesda Research Laboratories) containing either the hGR cDNA or β-actin cDNA was used to generate cDNA probes according to the procedure recommended by the supplier. Relative quantitation of bands on autoradiographs was performed using a GS500 transmittance scanning densitometer (Hoeffer Scientific Instruments, San Francisco, CA).

Chloramphenicol Acetyltransferase (CAT) Assays—Cell extracts were generated and CAT assays were performed essentially as described by Gorman et al. (28). The concentration of acetyl coenzyme A was increased to 1 mM, and the reaction times and concentration of [14C]Chloramphenicol were determined in preliminary studies to ensure that the percent conversion of [14C]Chloramphenicol to acetylated forms per micromgram protein was in the linear range of sensitivity. After autoradiography, radioactivity on thin layer chromatography plates were counted and counted to determine absolute levels of conversion. Protein concentrations were determined by the method of Lowry et al. (29).

Preparation of Labeled Glucocorticoid Receptor Extract and Southern Blotting—These procedures were done as previously described (30). Briefly, approximately 3 × 106 cells from a HeLa S3 suspension culture were incubated with 20 nM cortisol. After 24 h at 20°C, cells were harvested and the supernatant (cytosol) was collected following centrifugation at 100,000 × g for 1 h at 0°C. Cytosol, 1 ml, was added to a cocktail containing 50 nM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM dithiothreitol, and 4 μM unlabelled dexamethasone. After 1 h at 0°C, the mixture was filtered over a 0.45 μm filter and 20 μl were spotted onto a nitrocellulose filter as described by Maniatis et al. (27). Filters were hybridized with the recombinant plasmid pRSVCAT/ori. When indicated, poly(A)+-enriched RNA was obtained by binding and eluting RNA from oligo(dT)-cellulose. After autoradiography, radioactive spots on thin layer chromatography plates were counted and counted to determine absolute levels of conversion. Protein concentrations were determined by the method of Lowry et al. (29).

Isolation and Labeling of DNA Fragments—Restriction digests of plasmid DNAs were electrophoresed on 4% agarose gels, and bands containing the desired DNA fragments were excised and eluted.
as described in Maniatis et al. (27). The DNA fragments used for Southern blots were: (i) the 2.6-kb PstI-KpnI fragment from pRS5GR encompassing the entire hGR coding region; (ii) the 2.2-kb PstI-KpnI fragment from pRS6GR that contains no hGR cDNA sequences; (iii) the 844-bp AccI-ClaI fragment of pRS6GR that consists of a portion of the hGR coding sequence; and (iv) the 792-bp AccI-FspI fragment from pBR322 that lacks a GRE consensus sequence (33). Approximately 200 ng of each fragment (0.1-0.4 pmol) was labeled with [α-32P]dCTP using T4 DNA polymerase by replacement synthesis (34).

Western Blotting—Nitrocellulose filters containing immobilized cytosolic proteins were incubated at room temperature for 4 h in blocking buffer (10% nonfat dry milk, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Filters were then incubated overnight at 4 °C with rabbit antiserum raised against a peptide corresponding to a region within the immunogenic domain of the hGR, and then filters were washed in blocking buffer. 32P-Staphylococcal protein A (40 μCi) in blocking buffer was added to filters and incubated for 30 min; filters were washed and exposed to x-ray film.

RESULTS

Glucocorticoid Receptors Bind to the Human Glucocorticoid Receptor cDNA—Computer analysis of the hGR cDNA has revealed the presence of multiple sequences with homology to the GRE octamer that was originally identified by Payvar et al. (35) and to a putative negative GRE associated with the preproglucocorticoid hormone gene (36). Based on these observations, we wanted to determine whether these GRE-like elements, which reside within the glucocorticoid receptor gene, allow specific binding of receptor to its own cDNA. Such a result might suggest that these elements function in the regulation of GR gene expression by its cognate ligand. The ability of GR from HeLa cell cytosolic fractions to bind various fragments of DNA was analyzed by Southern blotting. This technique has been shown to permit analysis of specific GR protein-DNA interactions using cytosolic proteins immobilized on nitrocellulose (30). Only a minimal level of nonspecific protein-DNA interactions are seen with this procedure due to the inclusion of nonfat dry milk as a blocking agent during hybridization. The identity of GR on Southern blots can be verified by the capacity of the receptor to saturably bind to the affinity ligand dexamethasone mesylate. The pRS6GR KpnI-DraI fragment that contains the entire hGR cDNA coding sequence but lacks most of the 2.3-kb untranslated region at the 3' end (Fig. 1A, hGR) was bound in preference to two other DNA fragments, which consist either of vector sequences from pShGR (Fig. 1A, RSV) or a pBR322 fragment (Fig. 1A, PBR) that is devoid of GREs (33). Some binding of GR to the vector sequences in pRS6GR occurs and is most likely due to a sequence that matches the GRE consensus located in the pBR322 portion of the expression vector (bp 4044-4051 of pBR322) (33). Fig. 1A is an autoradiograph of an adjacent lane from the same gel that shows [3H]dexamethasone mesylate-bound GR comigrating with the [32P]DNA binding activity seen in panel A (at approximately 94-97 kDa). GR was saturably bound to [3H]dexamethasone mesylate as indicated by the absence of radiolabeled protein in the presence of excess unlabeled dexamethasone (data not shown).

Dexamethasone Treatment Results in Decreased Levels of hGR Protein in Transfected Cells—To establish whether GR binding to its cDNA affected regulation of GR gene expression, we analyzed the potential for transfected hGR protein levels to be down-regulated in response to hormone. Based on the observation that glucocorticoids down-regulate hGR protein and mRNA levels in a variety of cell lines and in rats (13, 14, 17), we wanted to determine if hGR that had been introduced into cells by transfection was also susceptible to down-regulation. We first determined that hGR from transfected cells resembled native GR both structurally and functionally. The cytosolic fraction isolated from COS cells transfected with the hGR cDNA expression vector, pRS6GR, was incubated with [3H]dexamethasone and subjected to sucrose density gradient centrifugation. This physical analysis confirmed that intact steroid binding hGR (9-10 S) was synthesized in these transfected cells (Ref. 37 and data not shown). To analyze for the function of hGR from transfected cells, a plasmid containing the glucocorticoid-sensitive mouse mammary tumor virus-LTR promoter linked to CAT was co-transfected with pRS6GR into COS cells. As expected based on the work of others (24), glucocorticoid-inducible CAT activity was seen in these cells indicating that the transfected hGR was able to activate transcription from a glucocorticoid-sensitive promoter (data not shown). We then analyzed the effect of dexamethasone on hGR levels in COS cells transfected with pRS6GR by two criteria: 1) immunoreactivity with anti-hGR antibodies and 2) binding to the radiolabeled affinity ligand [3H]dexamethasone mesylate. To demonstrate that GR protein levels were reduced in dexamethasone-treated transfected cells, cytosolic protein extracts from washed cells were analyzed by Western blotting. For this analysis we used antiserum generated against a peptide that is located in the immunogenic domain (NH2 terminus) of the hGR. Immune complexes were visualized following incubation of the immobilized proteins with 125I-staphylococcal protein A. The level of transfected immunoreactive hGR from cells grown in the absence of dexamethasone (Con) was greater than that from cells grown in the presence of dexamethasone (Dex) (Fig. 2A). To confirm that GR levels were reduced in dexamethasone-treated transfected cells, GR binding to [3H]dexamethasone mesylate was examined. Transfected cells were washed extensively to remove unlabeled dexamethasone (see "Materials and Methods") prior to incubation with [3H]dexamethasone mesylate. This wash procedure has been shown to remove greater than 98% of the unlabeled steroid and allows efficient labeling of GR from cytosols (9, 32). [3H]Dexamethasone mesylate-GR complexes (94-97 kDa) were visualized by fluorography of electrophoretically transferred proteins following NaDodSO4/PAGE. GR was identified by the specific and saturable binding to [3H]dexamethasone mesylate (labeling that was competed out in the presence of excess unlabeled dexamethasone) (Fig. 2B, compare lanes H ([3H]dexamethasone mesylate) and H+C).
Glucocorticoid Receptor Autoregulation

FIG. 2. Electrophoretic analysis of human glucocorticoid receptor protein in transfected cells. COS 1 cells were transfected with the hGR cDNA containing expression vector, pRShGR. Transfected cells were grown in the presence (Dex) and absence (Con) of dexamethasone. Cells were washed extensively and cytosolic extracts prepared. Proteins were subjected to NaDodSO₄-PAGE and electrophoretically transferred to nitrocellulose. All samples were loaded with equivalent amounts of protein (105 μg/lane, panel A and 300 μg/lane, panel B). A, Western blot analysis of GR. Filters were incubated with anti-human glucocorticoid receptor antisera followed by incubation with 125I-staphylococcal protein A and processed as described under “Materials and Methods” B, affinity labeling of human glucocorticoid receptor with [3H]dexamethasone mesylate. Transfected cells were washed extensively and incubated with [3H]dexamethasone mesylate alone (H) or together with 1000-fold molar excess of radioinert dexamethasone (H+C) and processed as described above. Filters were sprayed with ENHANCE and exposed to X-ray film. The locations of the prestained molecular weight markers (MYO, myosin (224,000 Da); PB, phosphorylase b (109,000 Da); and BSA, bovine serum albumin, (72,000 Da) is shown.

FIG. 3. Dexamethasone-mediated down-regulation of steady state human glucocorticoid receptor mRNA levels in transfected cells. COS 1 cells were transfected with pRShGR or mock-transfected and incubated with dexamethasone (Dex) or no additions (CON). Poly(A+) RNA was isolated, denatured, and separated on 1% agarose gels (2 μg/lane). RNA was then transferred to nitrocellulose and hybridized with [32P]-labeled hGR cRNA. The positions of the transfected hGR transcript (∼3.3 kb) and β-actin (∼2.8 kb) are indicated. The two experiments were performed independently and show the range of down-regulation observed.

([3H]dexamethasone mesylate + unlabeled dexamethasone). The amount of [3H]dexamethasone mesylate binding to transfected GR was greater from cells grown in the absence of dexamethasone (Con, lane H) compared with cells grown in the presence of dexamethasone (Dex, lane H) (Fig. 2B). The [3H]dexamethasone mesylate-labeled hGR comigrated with the immunoreactive hGR (Fig. 2). Both methods showed negligible levels of endogenous COS GR protein in mock transfected cells (data not shown).

Examination of hGR mRNA Levels from Transfected Cells—
To determine whether down-regulation of hGR protein was reflected at the level of mRNA we analyzed steady state hGR mRNA levels in response to hormone treatment. The hGR cDNA was transfected into COS cells, and total cellular RNA or poly(A+) RNA was isolated from control and dexamethasone-treated cells (36-40 h post-transfection). As shown in Fig. 3, Northern blot analysis revealed that hGR mRNA levels from transfected cells were markedly decreased in response to dexamethasone treatment. Only the transfected hGR mRNA (∼3.3 kb) encoded by pRShGR was present in these Northern blots, which is consistent with the observation that endogenous GR is not detectable in COS cells (24, 37). This transcript is smaller than the reported size of native GR transcripts due to removal from the hGR cDNA of most of the 3'-untranslated region, which contains the GR binding sites studied by Okret et al. (13). Fifteen independent experiments showed a mean decrease of 4-fold ± 0.5 S.E. in hGR mRNA levels following 12-17-h treatment with 2 x 10⁻⁷ M dexamethasone. The two blots in Fig. 3 are representative of the range of down-regulation that we observe. The blots were also hybridized with [32P]-labeled β-actin cDNA to confirm that equivalent amounts of RNA were present in control and dexamethasone-treated sample lanes. The differential signal intensities obtained in control and dexamethasone-treated samples were unaffected by the type of hybridization probe used, either [32P]-labeled hGR cDNA or cRNA.

Negatively Acting Sequences Are Located within the hGR cDNA—Expression of the hGR cDNA in pRShGR is driven by the RSV-LTR promoter contained on a 524-bp restriction fragment (24). Other investigators have shown that the RSV-LTR promoter is not regulated by dexamethasone (38, 39). To verify that the RSV promoter is not sensitive to dexamethasone and to rule out the possibility that other vector sequences in pRShGR influenced regulation by glucocorticoids, we examined CAT activity in cells cotransfected with the plasmids pRSVCAT/ori and pRShGR. Plasmid pRSVCAT/ori is identical to pRShGR except the hGR cDNA has been replaced by the cDNA encoding CAT. If sequences that are located outside the hGR cDNA were responsible for down-regulation of hGR mRNA levels, then CAT activity would be expected to decrease in response to dexamethasone treatment in cells that were transfected with pRSVCAT/ori. It was necessary to cotransfect cells with hGR since, as mentioned above, COS cells contain negligible levels of endogenous GR. As given in Table I, no difference in CAT activity in cell extracts prepared from control or dexamethasone-treated transfected cells was detected. This result demonstrates that the effects of dexamethasone on hGR mRNA levels observed in pRShGR- and pRSVCAT/ori transfected cells were specific for the hGR cDNA and were not due to the RSV promoter or to sequences present in the vector plasmid. As expected, transfection of pRSVCAT/ori alone (no cotransfected hGR) similarly resulted in no difference in CAT activity in response to dexamethasone (data not shown).

Down-regulation of Transfected hGR mRNA Is Time- and Dose-dependent—To determine if the glucocorticoid-induced decrease in hGR mRNA levels in transfected cells was a glucocorticoid receptor-mediated event we examined hGR mRNA levels following treatment with varying dexamethasone concentrations. The kinetics of this response were also examined. Treatment of hGR-transfected COS cells for 15 h with dexamethasone concentrations ranging from 1 x 10⁻¹⁰ to 1 x 10⁻⁴ M resulted in a dose-dependent down-regulation of hGR mRNA (Fig. 4). Data from two independent experiments are expressed as percentage of acetylation of [14C]chloramphenicol/25 μg of protein extract/t. The difference in CAT activity between experiments 1 and 2 is most likely due to variability in transfection efficiency. Dex, dexamethasone.

| Experiment | CAT activity |
|------------|--------------|
| +Dex       | -Dex         |
| 1          | 1.50         | 1.42         |
| 2          | 8.27         | 8.24         |

Table I

CAT activity from cells co-transfected with human glucocorticoid receptor and CAT expression vectors

* Cells were cotransfected with pRShGR and pRSVCAT/ori and treated as described under "Materials and Methods."

b Data are expressed as percentage of acetylation of [14C]chloramphenicol/25 μg of protein extract/t. The difference in CAT activity between experiments 1 and 2 is most likely due to variability in transfection efficiency. Dex, dexamethasone.
Glucocorticoid Receptor Autoregulation

### FIG. 4. Hormone concentration dependence of human glucocorticoid receptor mRNA down-regulation in transfected cells.

COS 1 cells transfected with pRSshGR were grown in the absence (Con) or presence of 1 × 10^{-8} to 1 × 10^{-6} M dexamethasone (Dex) for 17 h. Total cellular RNA was isolated, denatured, and separated on a 1% agarose gel (20 μg/lane). RNA was transferred to nitrocellulose and hybridized with 32P-labeled hGR cRNA. Arrows indicate hybridization to transfected hGR and to the 28 S ribosomal RNA (~4.7 kb).

### FIG. 5. Time course of human glucocorticoid receptor down-regulation by dexamethasone in transfected cells.

COS 1 cells transfected with pRSshGR were grown either in the presence (D) or absence (C) of dexamethasone (Dex) (2 × 10^{-8} M) for 10 min and 1, 2, 4, 6, 8, and 24 h. Total cellular RNA was isolated and examined on a Northern blot (inset) as described above. Hybridization of hGR mRNA in the absence and presence of dexamethasone was quantitated by densitometric scanning. The amount of hGR mRNA in the absence of dexamethasone was taken to be 100% of control.

### FIG. 6. Regulation of transfected DNA- and steroid-binding human glucocorticoid receptor mutants by dexamethasone.

COS 1 cells were transfected with hGR cDNAs that encode either a DNA binding mutant (DNA), a steroid-binding mutant (Steroid), or wild type receptor and grown in the presence (D) or absence (C) of dexamethasone. RNA was analyzed on Northern blots as described above.

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### Functional hGR Is Required for Down-regulation of hGR mRNA

Functional hGR is required for down-regulation of hGR mRNA—GR, like other members of the steroid receptor superfamily, contain discrete domains that are responsible for DNA binding, ligand binding, and transactivation functions (4). Data presented above suggest that down-regulation is receptor-mediated; to directly test this hypothesis we analyzed the ability of two GR mutants to down-regulate levels of hGR mRNA in response to dexamethasone. Transfections were performed using two hGR cDNAs mutants: one that encodes a non-DNA binding hGR and one that specifies a non-steroid binding variant. Both mutants were generated by insertion of linkers, which did not disrupt the protein reading frame (24). Fig. 6 shows that mRNA levels from either of these mutant receptors were not reduced in response to dexamethasone treatment. This result contrasts sharply with the results obtained for the wild type receptor and suggests that DNA- and steroid-binding functions of the GR receptors are required for down-regulation to occur and provides direct evidence that down-regulation is GR-mediated.

Down-regulation of hGR mRNA by the Glucocorticoid Antagonist RU486—RU486 binds with high affinity to glucocorticoid receptors; however, these RU486-GR complexes do not mediate transcriptional activation (41, 42). Based on these properties, we wished to determine whether down-regulation of hGR mRNA could proceed in transfected cells following RU486 treatment. This approach should help to reveal whether the transcriptionally active form of the receptor was required for down-regulation. Fig. 7 shows that hGR mRNA levels were reduced in response to RU486 (2 × 10^{-8} M) Concentrations between 10^{-8} and 10^{-7} M RU486 were sufficient to evoke a small (~25-30%) decrease in the levels of hGR mRNA as compared with untreated controls (data not shown). Treatment of transfected cells with steroids that interact with GR such as cortisol and progesterone caused down-regulation of hGR mRNA in contrast to estradiol (2 × 10^{-7} M), which had no effect on transfected hGR mRNA levels (data not shown).

Down-regulation of an hGR Deletion Mutant Is Impaired—To begin to localize sequences within the hGR cDNA that are responsible for down-regulation of transfected hGR mRNA, we evaluated the regulation of a deleted hGR cDNA. When an hGR cDNA that lacks sequences from bp +527 to +1526 (an AccI-ClaI fragment) was cotransfected with the intact hGR cDNA, levels of the truncated hGR transcript were partially decreased compared with the intact receptor mRNA (Fig. 8). We chose this region of the hGR for deletion because it contains a sequence with homology to the consensus GRE.

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mouse mammary tumor virus RNA by glucocorticoids (40). Together these data are consistent with the hypothesis that the glucocorticoid effects on hGR mRNA levels observed in transfected cells were mediated by GR.

Down-regulation is mediated by GR. This approach should help to reveal whether the transcriptionally active form of the receptor was required for down-regulation. Fig. 7 shows that hGR mRNA levels were reduced in response to RU486 (2 × 10^{-8} M) Concentrations between 10^{-8} and 10^{-7} M RU486 were sufficient to evoke a small (~25-30%) decrease in the levels of hGR mRNA as compared with untreated controls (data not shown). Treatment of transfected cells with steroids that interact with GR such as cortisol and progesterone caused down-regulation of hGR mRNA in contrast to estradiol (2 × 10^{-7} M), which had no effect on transfected hGR mRNA levels (data not shown).

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### FIG. 6. Regulation of transfected DNA- and steroid-binding human glucocorticoid receptor mutants by dexamethasone.

COS 1 cells were transfected with hGR cDNAs that encode either a DNA binding mutant (DNA), a steroid-binding mutant (Steroid), or wild type receptor and grown in the presence (D) or absence (C) of dexamethasone. RNA was analyzed on Northern blots as described above.
The Northern blot was hybridized with \("^P\)-labeled hGR cRNA. The were incubated with dexamethasone (DEX) or no additions (CON).

Fig. 7. Regulation of transfected human glucocorticoid receptor mRNA levels by RU486. COS 1 cells were transfected with pRShGR and grown in the presence of dexamethasone (Dex, 2 \( \times 10^{-7}\) M), RU486 (Ru, 2 \( \times 10^{-8}\) M), or were untreated (C). RNA was analyzed on Northern blots as described earlier, and signals on autoradiographs were quantitated by scanning densitometry. The average values and standard deviations of three experiments relative to untreated controls are shown.

that was derived from GREs from a number of glucocorticoid-regulated genes (4). Results of three independent experiments revealed that the levels of the truncated Acc-Cla hGR mRNA were reduced to 48 ± 5% whereas intact hGR mRNA levels were decreased to 26 ± 3% of control levels. This result suggests that the region between nucleotides +527 and +1526 of the hGR cDNA contains at least some of the signals that are necessary for down-regulation. Southwestern blotting revealed that hGR from HeLa cytosol bound to the 844-bp hGR Acc-ClaI restriction fragment (contained within the 1000-bp Acc-ClaI fragment) in preference to an equivalently sized non-GRE-containing fragment of pBR322 (Fig. 9A). Panel 9B confirms that \([^{\text{32}}\text{P}]\)dexamethasone mesylate-labeled GR comigrated with the \([^{\text{32}}\text{P}]\)DNA binding activity seen in Fig. 9A. The higher molecular weight \([^{\text{32}}\text{P}]\)DNA binding ac-

Fig. 8. Regulation of cotransfected, truncated, and wild type human glucocorticoid receptor mRNA levels by dexamethasone. Total RNA was prepared from cells cotransfected with pBRhGR and \(\Delta\)Acc-Cla hGR (hGR lacking bp +527 to +1526). Cells were incubated with dexamethasone (DEX) or no additions (CON). The Northern blot was hybridized with \("^P\)-labeled hGR cRNA. The transcripts from pBRhGR and \(\Delta\)Acc-Cla hGR are -3300 and -2300 nucleotides, respectively. Hybridization to \("^P\)-labeled \(\beta\)-actin is shown at the bottom of the figure.

We have examined steady state hGR mRNA and protein levels from COS 1 cells following transfection with an hGR expression vector. Down-regulation of hGR mRNA and protein levels was observed in transfected cells in response to glucocorticoid treatment. Non-hGR sequences located in the vector were not responsible for the glucocorticoid-mediated alteration in receptor levels; thus, it appears that the hGR cDNA contains sequences sufficient to evoke the glucocorticoid-induced down-regulation of hGR mRNA and protein seen in transfected cells. This effect required the DNA- and steroid-binding functions of the receptor as demonstrated by the failure of mRNA from either a DNA- or a steroid-binding deficient mutant to be down-regulated. Moreover, the time and dose dependence of down-regulation by dexamethasone in transfected cells provides further evidence that this effect is receptor-mediated.

There was some variability observed in the extent of down-regulation of hGR mRNA in response to dexamethasone between the different experiments. We attribute this variability to differences in transfection efficiencies and, perhaps to a lesser extent, to endogenous steroid in the cell culture media and to slight differences in the times of exposure to dexamethasone. Nevertheless, down-regulation was consistently observed regardless of variations in the initial levels of hGR mRNA.

We do not know if down-regulation of mRNA levels in transfected COS 1 cells is due to transcriptional or post-transcriptional effects. It has been reported that the rate of endogenous GR transcription is decreased in dexamethasone-treated human IM9 lymphocytes (17) and in rat liver (15). In addition, Rosewicz et al. (17) and Dong et al. (15) failed to see any dexamethasone effects on GR mRNA half-life in cultured cells. The kinetics of down-regulation that we observed for the transfected hGR mRNA resembled that observed for the endogenous GR mRNA (17). Also, transfected hGR mRNA displayed a sensitivity to varying dexamethasone concentra-
tions that was similar to endogenous GR mRNA (17).

We have shown that treatment with RU486 (a glucocorticoid antagonist) resulted in down-regulation of hGR mRNA in transfected cells; however, a 5-fold higher dose of RU486 was used. Down-regulation of endogenous GR mRNA by RU486 was demonstrated in NIH3T3 cells (16) and in the rat brain (43) but was not seen in the human lymphoblastoid IM-9 cell line when a 10-fold lower concentration of RU486 was used (44). Since it has been shown that RU486-GR complexes bind DNA with a specificity that is similar to dexamethasone-GR complexes (44), it is unreasonable that this antagonist elicited down-regulation. These effects of RU486 suggest that the transcriptional activating function of the receptor may play a major role in down-regulation although a partial agonist function of RU486 cannot be excluded. That down-regulation was seen following treatment with RU486 argues that this phenomenon is not due to glucocorticoid activation of a transcriptional repressor in transfected cells.

By analogy with positively regulated genes, inhibition of gene expression by glucocorticoids may involve binding of receptor to cis-acting sequences. GREs have been reported in various locations within or associated with genes that are positively regulated by glucocorticoids. The locations of these GREs vary from a few kilobases upstream from the promoter (e.g. tyrosine aminotransferase gene (45)) to within the first intron of the growth hormone gene (46). In contrast, regulatory regions for genes whose transcription is negatively regulated by glucocorticoids (e.g. glucocorticoid receptor (47), and prolactin (39, 51)) have only been reported to date in regions that are 5' to the promoters of the various genes. A weak consensus sequence has been identified in some genes whose transcription is inhibited by glucocorticoids. This putative negative GRE (nGRE) does not resemble the consensus GRE associated with genes that are positively regulated by glucocorticoids (5). It should be noted that this partial nGRE consensus is not contained within the hGR cDNA; however, some sequences with homology to a potential nGRE in the pro-opiomelanocortin promoter region (36) are located within the Acc-Cla fragment of the hGR. Perhaps autoregulatory signals are present within the 1-kb Acc-Cla fragment of the hGR cDNA since deletion of this region yielded a truncated hGR mRNA that was somewhat refractory to down-regulation. In addition, GR from HeLa cytosol bound to this fragment in preference to a pBR322 restriction fragment, further supporting a possible regulatory interaction between GR and its gene or perhaps with its mRNA. These putative intragenic regulatory sequences in the hGR could potentially function transcriptionally or post-transcriptionally in the down-regulation of receptor. We are currently working to identify the precise autoregulatory signals within the hGR cDNA and understand their mechanism of action.

Acknowledgments—We thank Drs. Ron Evans, Stanley Hollenberg, and Frank Rutter for providing us with plasmid constructs. The editorial assistance of Deborah Bellingham is greatly appreciated.

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