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Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone

(gene regulation/autoregulation/negative feedback/protein–DNA interaction/cycloheximide)

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ABSTRACT **A cDNA clone for the rat glucocorticoid receptor (GR) was used to study mechanisms of GR mRNA regulation. Treatment of rat hepatoma culture cells with 0.5 μM dexamethasone caused a small, initial increase in the GR mRNA level after 6 hr as well as a 50% to 95% reduction of the GR mRNA level after 24 hr of incubation when studied by RNA blot hybridization. After 72 hr, the initial GR mRNA level was restored. The down-regulation of GR mRNA levels appears to be independent of protein synthesis, since it also was observed in the presence of cycloheximide. However, cycloheximide caused a 4-fold increase in intracellular levels of GR mRNA. Using an immunoprecipitation assay, we could demonstrate that the GR specifically interacts with a GR cDNA clone, which represents a 2.6-kilobase fragment of the 3′ nontranslated region of the GR mRNA. Nuclease protection experiments indicate the presence of several internal GR-binding regions in the above fragment.**

Steroid hormones act by binding to an intracellular receptor protein. Subsequently, the hormone–receptor complex interacts with the genome and regulates transcription of selected genes in either a stimulatory or inhibitory manner (for reviews, see refs. 1 and 2). *In vitro*, purified glucocorticoid receptor (GR) has been shown to recognize specific sequences in genes that are positively regulated by glucocorticoid hormones *in vivo* (2–7). These sequences have been shown to act as GR-dependent transcriptional enhancer elements *in vivo* (7, 8). Therefore, a prerequisite for a glucocorticoid response in the cell is the presence of GR (9). However, this does not always guarantee hormonal sensitivity in a GR-containing cell, since there is a great variation in glucocorticoid sensitivity due to cell differentiation (10), absence of a functional receptor (11), receptor-modifying factors (12), and receptor concentration (13, 14). In case of receptor concentration, cellular GR levels have been shown to vary as a result of endocrine manipulations (15–18), neural influence, and cell contact (19, 20) during different stages in the cell cycle (21) and during aging (22).

Autoregulation of hormone-receptor concentration by the receptor ligand as determined by ligand-binding or immunochemical assays is well documented for a number of peptide, sterol, and neurotransmitter receptors (for a review, see ref. 23). In case of the low density lipoprotein receptor, a down-regulation by sterols has also been shown to be reflected in the mRNA level (24). Autoregulation of steroid receptors has been indicated by several investigators using ligand binding assays. For instance, the presence of glucocorticoids has been reported to cause a down-regulation of GR (15–17). Adrenalectomy gives rise to an up-regulation of the cellular GR concentration (18). However, in receptor assays using ligand binding, an accurate quantitation requires a total exchange of receptor-bound unlabeled steroids (natural or administered) for a labeled tracer. In many cases this requires various manipulations of receptor preparations with unknown consequences for the receptor protein. Furthermore, GR seems to exist in two stages concerning the ability to bind ligand (12), which might hamper receptor quantitation based on a ligand binding assay. In this report, we utilize a GR cDNA probe to evaluate changes in GR mRNA levels after various endocrine manipulations. We show that glucocorticoid administration is accompanied by a decrease in cellular GR mRNA. GR is also shown to recognize specific sequences within a fragment of the 3′ nontranslated GR cDNA.

MATERIALS AND METHODS

Cells and Animals. Rat Rueber hepatoma cells (H4IIE, hepatoma tumor cell line HTC) were obtained from the American Tissue Culture Collection and grown in RPMI 1640 medium (Flow Laboratories) supplemented with 8% (vol/vol) heat-inactivated and charcoal-treated (1 hr, 37°C) fetal calf serum (GIBCO). 2 mM L-glutamine (Flow Laboratories), bensylpenicillin (400 international units per ml; Astra, Södertälje, Sweden), streptomycin (0.2 mg/ml; Novo, Copenhagen) and, when indicated, 0.5 μM dexamethasone (Sigma) and/or cycloheximide (Sigma) at 1.5 μg/ml. Eight-week-old male Sprague–Dawley rats were used for *in vivo* experiments.

Recombinant Clones. pRM16 contains a 2.6-kilobase (kb) GR cDNA insert corresponding to the far 3′ nontranslated end of the ≈7-kb GR mRNA (25, 26) in the pCD vector (27). The GR cDNA insert was also cloned into the pSP65 vector to produce a cRNA probe by SP6 polymerase (28). pA_2 contains a 4.7-kb *Eco*RI insert of the mouse mammary tumor virus (MMTV) genome containing binding regions 1–4 in the pML2 vector, a pBR322 derivative (29). A cDNA probe for β-actin was from Cleveland *et al.* (30).

Preparation and Blot-Hybridization Analysis of RNA. RNA was prepared by using the guanidinium thiocyanate method (31) and centrifuged through 5.7 M cesium chloride. Total RNA (20 μg per lane) was analyzed in 0.9% (wt/vol) agarose-formaldehyde gels, blotted to nitrocellulose sheets and hybridized as described (32). Hybridization was performed with 3×10^6 cpm of the pRM16 cRNA probe per ml for 16 hr at 60°C. Washing was performed four times for 5 min at room temperature, followed by two washes for 60 min at 65°C in 30 mM NaCl/3 mM sodium citrate, pH 7.0/0.1% sodium dodecyl sulfate. In cases where nick-translated cDNA probes were used, hybridization was performed at 50°C, followed by washing at 55°C. Relative optical density

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Abbreviations: GR, glucocorticoid receptor; bp, base pairs; kb, kilobase(s); HTC, hepatoma tumor cell line; MMTV, mouse mammary tumor virus.

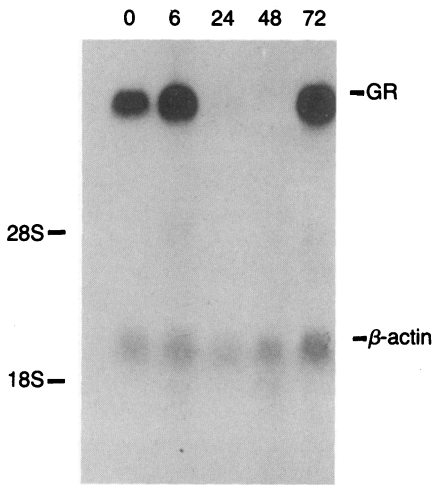

FIG. 1. RNA blot-hybridization analysis of GR mRNA in HTC cells after dexamethasone treatment for 0–72 hr as indicated at the top of the lanes. Total RNA (20 μg per lane) from HTC cells was separated on 0.9% agarose-formaldehyde gels, blotted onto nitrocellulose filters, and hybridized with pRM16 cRNA and β-actin cDNA probes, respectively. HTC cells were cultured for 72 hr in the presence of 0.5 μM dexamethasone. The positions of 28S and 18S ribosomal RNA were determined by staining parallel lanes with acridine orange. Only slight changes (15–20%) in β-actin mRNA levels were observable.

of the bands was determined by scanning the autoradiographs in a Beckman R-112 densitometer at 550 nm.

Receptor–DNA Interaction and Immunoprecipitation. pRM16 and pA_2 were digested with restriction endonucleases *Pst* I, *Xho* I, and *Eco*RI (Boehringer Mannheim), respectively. The obtained fragments were end-labeled with [α-^{32}P]dATP (3000 Ci/mmol, Amersham; 1 Ci = 37 GBq) by using the *Escherichia coli* large (Klenow) fragment of DNA polymerase I (Bethesda Research Laboratories). Labeled DNA (10 ng) was incubated with 100 ng of purified rat liver GR (33) for 15 min at room temperature in 0.25 ml of binding buffer (20 mM Tris·HCl, pH 7.8/60 mM NaCl/2 mM $MgCl_2$/1 mM EDTA/20% (wt/vol) glycerol/250 μg of insulin per ml/2 mM dithiothreitol/1 μM unlabeled triamcinolone acetonide). After a 15-min incubation at room temperature, 1 M $MgCl_2$ and salmon sperm DNA at 1 mg/ml were added to a final concentration of 15 mM and 40 μg/ml, respectively, and incubated for an additional 15 min at room temperature to decrease nonspecific DNA-binding (29). Thereafter, 50 μl of 10% (vol/vol) *Staphylococcus aureus* (Pansorbin, Calbiochem–Behring) coated with saturating amounts of protein A-purified monoclonal anti-GR antibody (no. 7, ref. 34) was added and incubated for an additional 30 min at room temperature with end-to-end rotation. The whole incubation mixture was applied to a 0.8-ml cushion of 1 M sucrose in washing buffer (20 mM Tris·HCl, pH 7.4/0.1 mM EDTA/10 mM dithiothreitol) and centrifuged for 5 min at 10,000 × *g*. After three washes of the pellet in washing buffer without sucrose, the DNA was extracted with 0.1% sodium dodecyl sulfate in 20 mM Tris·HCl, pH 7.4/0.1 mM EDTA/20 mM NaCl for 30 min at 65°C, precipitated with ethanol, and analyzed by 1% (wt/vol) agarose gel electrophoresis and autoradiography. For DNase I protection experiments, DNA was end-labeled as described above. In each reaction, ≈0.5–1 nM of labeled DNA was incubated in the presence or absence of receptor (0–20 nM) in 100-μl assays and digested with DNase I (grade I, Boehringer Mannheim) as described (3). All cleavage products and the corresponding Maxam–Gilbert sequence markers (35) were analyzed on denaturing 8% (wt/vol) polyacrylamide gels and visualized by autoradiography.

RESULTS

To investigate the effect of dexamethasone on cellular GR mRNA levels, HTC cells were grown in a logarithmic phase for various times in the absence or presence of 0.5 μM dexamethasone, whereafter GR mRNA was analyzed by RNA blot-hybridization analysis (Fig. 1). A reduction by a factor of ≈15 in the amount of a single ≈7-kb GR mRNA species was seen in this experiment at 24–48 hr after treatment with dexamethasone. This decrease occurred after a slight (≈2-fold) but significant increase in the GR mRNA level after 6 hr of incubation. Although the down-regulation of GR mRNA after hormone treatment for 24 hr was a consistent observation in all experiments, the degree of down-regulation caused by treatment with 0.5 μM dexamethasone varied between 50% and 95%. After 72 hr of incubation, the initial level of GR mRNA was restored (Fig. 1). The changes in the amount of GR mRNA were evaluated in relation to the same amount of total cellular RNA and to the content of β-actin mRNA, which only exhibited slight changes (15–20%) upon hormone treatment (Fig. 1). A similar degree of down-regulation (50% to 90%) of cellular GR mRNA was also seen *in vivo* in livers of rats treated with 4 mg of dexamethasone per kg of body weight for 4 days (data not shown).

To investigate the importance of protein synthesis for down-regulation of GR mRNA by dexamethasone, experiments in HTC cells were performed as above in the absence or presence of the inhibitor of translation, cycloheximide.

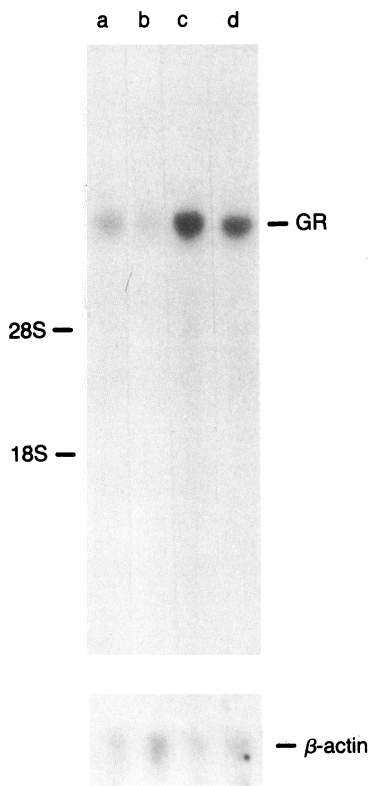

FIG. 2. RNA blot-hybridization analysis of GR mRNA in HTC cells after dexamethasone treatment in the presence or absence of cycloheximide. Total RNA (20 μg per lane) from HTC cells was analyzed as described in the legend to Fig. 1. HTC cells were grown under serum-free conditions for 18 hr in medium containing neither dexamethasone nor cycloheximide (lane a), dexamethasone but no cycloheximide (lane b), cycloheximide but no dexamethasone (lane c), and both dexamethasone and cycloheximide (lane d). The concentration of dexamethasone was 0.5 μM. Cycloheximide was added to a final concentration of 1.5 μg/ml 1 hr before addition of dexamethasone.

Cycloheximide was added to the cultures (final concentration, 1.5 μg/ml) 1 hr before addition of dexamethasone. This concentration of cycloheximide inhibited >95% of the incorporation of [^{35}S]methionine into cytosolic protein precipitable by trichloroacetic acid, as measured during an 18-hr period (data not shown). In these experiments, control cells grown in the absence of cycloheximide showed 50% reduction of GR mRNA after 18 hr of incubation with 0.5 μM dexamethasone (Fig. 2, lanes a and b). However, incubation of HTC cells with cycloheximide at 1.5 μg/ml caused an ≈4-fold increase in GR mRNA (Fig. 2, lane c) as compared to cells grown in the absence of cycloheximide (Fig. 2, lane a). Although the amount of GR mRNA was higher in cells grown in the presence of cycloheximide, a reduction by a factor of ≈2 (50%) of GR mRNA also occurred in the presence of cycloheximide after treatment with dexamethasone (Fig. 2, lane d). This decrease is in the same range as the decrease of GR mRNA observed in the control incubations upon addition of dexamethasone but in the absence of cycloheximide. Neither cycloheximide nor dexamethasone significantly affected the levels of β-actin mRNA (Fig. 2, lower panel).

The experiments described above indicate that down-regulation of GR mRNA by dexamethasone might be independent of translation. Since GR–DNA interaction appears to be an essential step in mediating the hormone response (see above), we investigated whether the GR can recognize specific sequences in the GR cDNA. The GR cDNA clone pRM16 represents 2.6 kb of the far 3′ nontranslated end of the 7-kb GR mRNA. This region exists in the GR genome as a part of a single exon (26). pRM16 was digested with restriction nucleases *Pst* I and *Xho* I, giving rise to a mixture of the 2.6-kb GR cDNA insert and the three vector fragments (1.8, 0.8, and 0.2 kb; see Fig. 3*A Upper*), which were end-labeled and incubated with purified rat liver GR. GR–DNA complexes were isolated by precipitation with monoclonal anti-GR antibodies, and the labeled DNA was analyzed by electrophoresis. Fig. 3*A*, lane c, shows a clear selectivity of GR binding to the GR cDNA as compared to the vector fragments (compare with Fig. 3*A*, lane a, which shows the input DNA prior to immunoprecipitation). The selectivity was still valid when the different nucleotide lengths of the GR cDNA and vector fragments were taken into account. When control antibodies from ascites fluid of mice injected with the nonimmunoglobulin-producing mouse myeloma cell line Sp2/0 (34) were used instead of anti-GR antibodies, almost no DNA was precipitated (Fig. 3*A*, lane d). No DNA bound unspecifically to Pansorbin (Fig. 3*A*, lane b). However, immunoprecipitation was dependent on the presence of GR, since monoclonal anti-GR antibodies in the absence of GR did not precipitate any DNA (Fig. 3*A*, lane e). As a control for the specific recognition of GR cDNA sequences by GR in the immunoprecipitation assay, a comparison was made to pA_2, a clone containing 4.7 kb of the MMTV genome (29). This clone harbors four previously defined GR binding regions (3, 29). A selective recognition of GR for MMTV DNA as compared to the vector fragment (pML2) was seen with this assay (Fig. 3*B*), in agreement with results obtained with nitrocellulose filter binding experiments (29).

The degenerate consensus octanucleotide 5′ AGA$\frac{A}{T}$CA(G)-$\frac{A}{T}$ 3′ has been derived from GR binding regions in MMTV (3) and also has been found to occur in the GR cDNA clone pRM16 (52). To test the hypothesis if these regions may be recognized by the receptor, a 245-base-pair (bp) *Hin*dIII–*Stu* I fragment of pRM16 located ≈1.1 kb upstream of the poly(A) tract and containing three motifs with 87.5% homology of the above consensus sequence was chosen for DNase I protection experiments. Low concentrations of purified GR (7.5–15 nM) protected two regions with different degrees of protection (Fig. 4). The best protection was found in a 97-bp segment (site 1) with two 3′ consensus motifs in close proximity to each other and extending 63 bp 5′ to the consensus motifs along the coding strand. Weaker protection was observed within a 21-bp segment (site 2) containing a third motif with a similar match to the consensus sequence as the two motifs above (i.e., 87.5%).

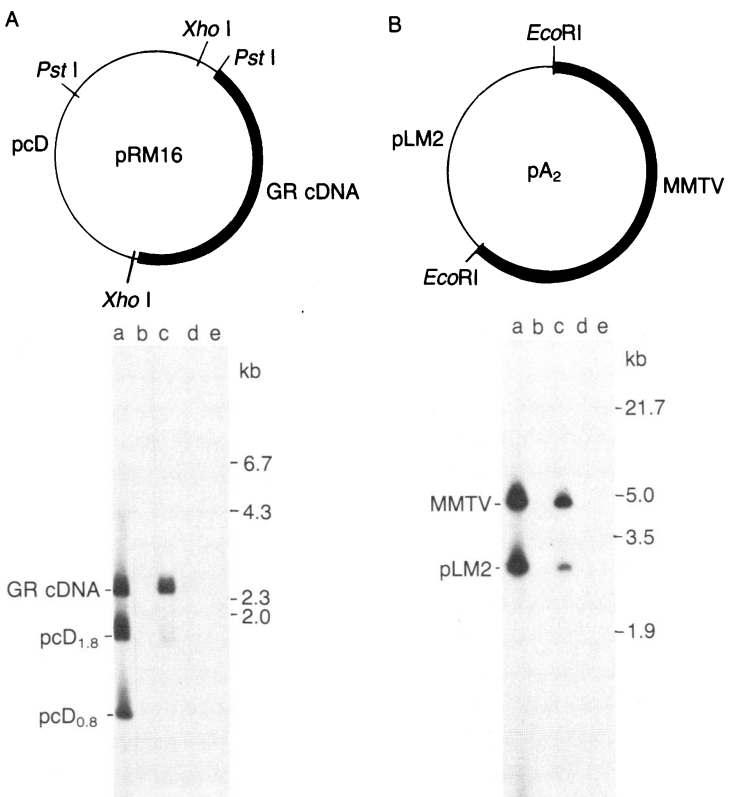

FIG. 3. Selective binding of purified rat liver GR to GR cDNA and MMTV DNA. (*A*) Clone pRM16, consisting of 2.6 kb of the far 3′ sequence of GR cDNA (bold line) in the 3.2-kb pcD vector was digested with restriction endonuclease *Xho* I and *Pst* I. The fragments formed, the 2.6-kb GR cDNA insert, and the three pcD vector fragments (1.8, 0.8 and 0.2 kb, respectively), were end-labeled and subjected to receptor binding experiments. DNA fragments that bound purified rat liver GR were immunoprecipitated with monoclonal anti-GR antibodies and analyzed by 1% agarose gel electrophoresis and autoradiography. Lanes: a, DNA prior to immunoprecipitation; b–e, precipitated material: in the absence of antibody (lane b), in the presence of monoclonal anti-GR antibodies (lane c), in the presence of control antibodies (lane d), and in the presence of monoclonal anti-GR antibodies but in the absence of GR (lane e). The 0.2-kb pcD vector fragment is not seen on the gel. (*B*) The clone pA_2, consisting of a 4.7-kb *Eco*RI insert of MMTV (bold line) with GR binding regions 1–4 in vector pML2, was digested with restriction endonuclease *Eco*RI. The formed fragments, the 4.7-kb MMTV DNA, and the 3.0-kb pML2 vector were end-labeled, subjected to receptor binding experiments, and analyzed as above. For explanation of the different lanes, see *A*.

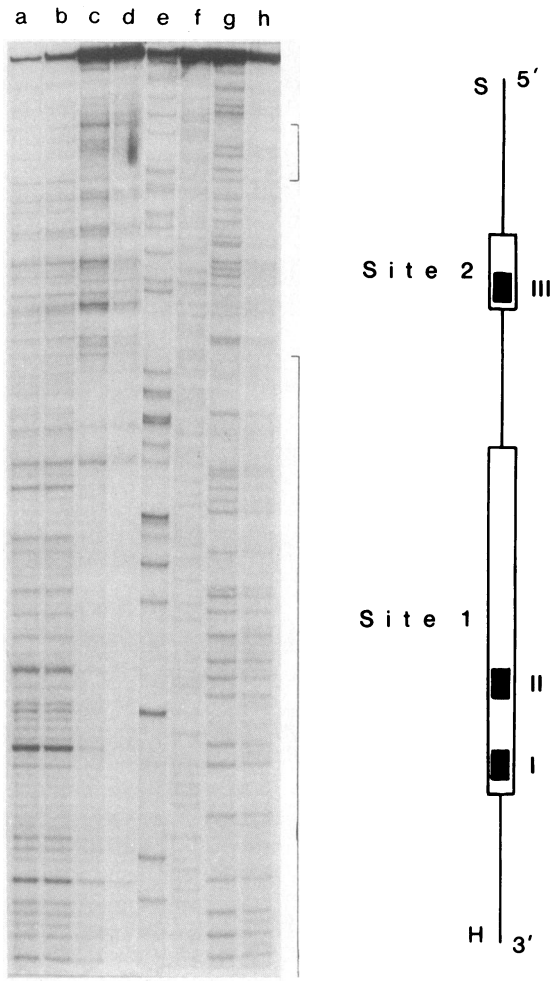

FIG. 4. DNase I-cleavage protection experiments of GR bound to DNA sequences within the 3′ noncoding flank of the GR cDNA. The coding strand of a 245-bp *Hin*dIII–*Stu* I fragment of pRM16 was end-labeled at the upstream *Hin*dIII site located ≈1.1 kb upstream of the poly(A) tract. DNA (0.1 pmol) was incubated with 0 (lane a), 25 (lane b), 75 (lane c), or 150 ng of GR (lane d). The autoradiogram displays DNase I-cleavage products (lanes a–d) as well as G-, G+A-, C-, and T+C-sequencing reactions (lanes e–h). The diagram schematically represents the 245-bp fragment. □, GR-binding consensus motifs; □, area protected by GR; H, *Hin*dIII; S, *Stu* I.

DISCUSSION

Using exchange-dependent ligand binding assays, several investigators have demonstrated a down-regulation of cellular GR levels after hormone treatment (15–17). In this report, we show that glucocorticoid hormone-treatment for 24–48 hr results in a down-regulation of cellular GR mRNA levels both in HTC cells and in rat liver *in vivo*. However, this down-regulation is transient because the GR mRNA level appears to be restored after 72 hr. Furthermore, an initial increase in GR mRNA was observed before the down-regulation occurred. At the present time, it is not understood whether these observations reflect changes during the various cell cycle phases. Such changes, in turn, may be caused by hormone treatment, although the cells were maintained in logarithmic growth. In all RNA blot-hybridization experiments, a single ≈7-kb GR mRNA species was recognized by pRM16. By using other GR-specific cDNA probes, additional GR mRNA species have been described in several cell lines (26, 36, 37). The changes in GR mRNA levels could be due to effects on transcription and/or turnover rate of receptor mRNA, both of which represent target levels of gene regulation by steroid hormones (2, 38). Additional posttranscriptional regulatory mechanisms cannot be excluded. For instance, an increased depletion of GR without any change in receptor synthesis rate has been suggested after treatment with glucocorticoids (16, 39).

Biochemical as well as genetic experiments have indicated the importance of the GR in controlling the expression of both positively and negatively regulated genes (11, 40). The glucocorticoid-caused down-regulation of the GR transcript observed in the presence of the inhibitor of protein synthesis, cycloheximide, indicates that this response might not be dependent on the synthesis of a putative second *trans*-acting factor but directly mediated by the GR protein itself. In case of positively regulated genes (e.g., MMTV), the GR has been shown to recognize specific DNA contact regions *in vitro* that represent positive glucocorticoid response elements *in vivo* (2). Interestingly, GR-specific contact regions have been described not only upstream of the MMTV promoter in the 5′ long terminal repeat but also within discrete regions far from the promoter and within transcribed and translated portions of the MMTV element (3). An internal binding region for GR also has been demonstrated in the human growth hormone gene, which is positively regulated by glucocorticoids (6, 7). Furthermore, the interaction of regulatory proteins other than the GR with internal binding sites has been described in, for instance, the 5S RNA and immunoglobulin genes (41–45); and *cis*-acting regulatory sequences have been shown to be located within, for instance, the β-globin gene (46, 47). Our findings using an immunoprecipitation technique for analyzing GR–DNA interaction support the contention of GR-specific binding region(s) within the GR cDNA clone pRM16. This observation was further substantiated by DNase I-cleavage protection analysis of a 245-bp fragment of pRM16. This particular fragment contains three copies (≧ 87.5% homology) of the degenerate consensus sequence, 5′ AGA$^{A}_{T}$CA(G)$^{A}_{T}$ 3′, previously emphasized for specific GR–DNA interaction in, for instance, MMTV (3, 4). These motifs are also included in each of the protected regions within the pRM16 fragment. In addition, binding site 1 extends 63 bp downstream of the consensus motif, indicating a possible divergence from GR binding sites in positively regulated genes. It is tempting to speculate that down-regulation of GR mRNA by glucocorticoid hormones is caused by interaction of the GR protein with its own gene in analogy to the postulated model for positive gene regulation by glucocorticoids (cf. ref. 2) or, alternatively, by interaction of the receptor with its own preexisting and/or newly synthesized mRNA. Clearly, it remains to be established that the internal GR binding region described in this report is functional in the regulation of GR mRNA levels *in vivo*.

In the presence of the inhibitor of protein synthesis, cycloheximide, GR mRNA levels were increased ≈4-fold (Fig. 2). Whether this is the result of a stabilization of the GR mRNA or due to the inhibition of synthesis of a repressive *trans*-acting factor is not yet clear. However, caution must be exercised in the interpretation of experiments involving the use of metabolic inhibitors. It is known that cycloheximide produces a nonspecific stabilizing effect on polysomal RNA (48). In contrast to a reported ≈2-fold induction of β-actin mRNA by cycloheximide (49), little, if any, effect of cycloheximide on β-actin mRNA levels were observed under our experimental conditions. In this context, it is interesting to note that the control of the expression of a specific isozyme of cytochrome P-450, P-450c, appears to be mediated by at least two *trans*-acting factors (50): (*i*) the dioxin receptor, a positive regulator, the properties of which are strikingly similar to steroid receptors (51); and (*ii*) a negative regulatory factor, the expression of which is suppressed by cycloheximide.

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