Regulation of Glucocorticoid Receptor Expression: Evidence for Transcriptional and Posttranslational Mechanisms

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The mechanism of ligand-induced (homologous) down-regulation of the glucocorticoid receptor (GR) has been studied. Dexamethasone caused a down-regulation of the levels of GR mRNA and protein both in hepatoma tissue culture cells and rat liver *in vivo*. The decrease in the level of rat liver GR mRNA was due to a reduced transcription rate of the GR gene, as assessed by nuclear run-on transcription experiments. The half-life of GR mRNA in hepatoma tissue culture cells was determined to be approximately 4.5 h and was unaffected by dexamethasone. In addition to the transcriptional regulation of GR gene expression, a dexamethasone-dependent posttranslational modification in the rate of GR protein turnover was observed. In the absence of dexamethasone, GR protein half life was approximately 25 h whereas it decreased to approximately 11 h in the presence of hormone. Down-regulation of GR protein occurred with a 6- to 24-h delay as compared to the decline in GR mRNA. This is most likely due to the differences in half-lives of GR mRNA and protein, respectively. Our results suggest that autoregulation of GR by its cognate ligand is complex and occurs at both transcriptional and posttranslational levels. (Molecular Endocrinology 2: 1256–1264, 1988)

INTRODUCTION

Steroid hormone receptors are soluble intracellular proteins active as transcription factors mediating the biological effects of steroids by regulating gene expression at a predominantly transcriptional level. This regulation is believed to occur through specific interaction of hormone-receptor complexes with hormone-responsive enhancer elements in target genes (for reviews, see Refs. 1 and 2). The presence of a functional receptor is therefore a prerequisite for the biological response of the hormone. In most systems, although not in all (3), a relationship between steroid receptor concentration and degree of biological response has been demonstrated. For instance, early work (4, 5) has indicated a relationship between nuclear glucocorticoid binding and effect, implicating the limiting nature of the receptor in the response. Bourgeois and Newby (6) showed a correlation between cellular glucocorticoid sensitivity and concentration of glucocorticoid receptor (GR) by fusing mouse lymphoid cells with various concentrations of GR. A relationship, although not perfect, has also been observed between cellular GR content and glucocorticoid response in individuals suffering from leukemia (7). Furthermore, Danielsen and Stallcup (8) isolated a cell variant, mouse thymoma W7, that had overcome its inherited sensitivity to glucocorticoids by reversibly down-regulating its GR concentration, supporting the notion that the GR level is important in determining the degree of the cellular response. Recently, Vanderbilt *et al.* (9) studied an initially receptor negative cell line after stable transfection with a GR expression vector and was able to show that the magnitude of several transcriptional responses elicited by GR is approximately proportional to the number of receptor molecules expressed in the cell. This suggests that the GR is the limiting factor in the response cascade and, furthermore, that small changes in the receptor concentration will be reflected in parallel changes in the cellular sensitivity to glucocorticoid hormones. It is therefore of interest to determine mechanisms and trans-acting factors involved in regulation of GR expression.

Using ligand binding techniques, it has been shown that steroid hormones autoregulate their own receptors (for reviews, see Refs. 10 and 11). Treatment of target cells with glucocorticoids causes a 50–80% down-regulation of cellular GR (12–14). In line with this, ad-

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renalectomy causes an approximate 2-fold increase in GR concentration in certain tissues (15). The glucocorticoid induced down-regulation of GR is a relatively slow response requiring more than 18 h to reach maximal effect. Furthermore, the process is independent of ongoing protein synthesis suggesting that down-regulation of GR caused by glucocorticoid hormones is mediated by the receptor itself (14, 16). We have previously shown that glucocorticoid treatment of hepatoma tissue culture (HTC) cells causes a down-regulation of GR mRNA and that this down-regulation does not require ongoing protein synthesis (17). However, the regulation of GR mRNA appears complex in that the GR mRNA level initially increases before its decline.

Expression of a functional protein may be regulated at both transcriptional and posttranscriptional levels, the latter including effects on, *e.g.* mRNA stability, translation efficiency, protein turnover, or conversion of the protein from a nonactive to an active form. In the present study, we have extended our investigations referred to above with the aim to determine at what level(s) GR autoregulation occurs. We conclude that the regulation of GR expression is determined by both transcriptional and posttranslational processes.

RESULTS

Autoregulation of GR Protein by Dexamethasone in HTC Cells

We have previously shown that dexamethasone affects GR mRNA levels in HTC cells (17). The mechanism of regulation of GR appears complex since treatment of the cells with dexamethasone results in an initial 2-fold increase in GR mRNA after 6 h followed by a decrease to approximately 20% of the original GR mRNA concentration after 24–48 h. In order to investigate if the changes in GR mRNA levels were followed by corresponding changes in GR protein levels, HTC cells were grown under identical conditions as before (logarithmic growth with 0.5 μM dexamethasone) and GR protein levels were analyzed after various times by Western immunoblotting using a monoclonal anti-rat GR antibody. An initial approximately 2-fold (range, 1.5 to 2.5-fold in four separate experiments) increase in GR immunoreactivity was seen after 12 h of dexamethasone treatment compared to untreated cells (the sum of the 94 and 91 kilodalton (kDa) bands, see below) (Fig. 1). After 48 h, the GR protein level had declined to approximately 20–40% of the level found in untreated cells. Thus, the changes in GR mRNA levels after dexamethasone treatment are reflected in corresponding changes in GR protein levels, although the latter occurred with a 6- to 24-h delay. Actin protein did not change significantly during the treatment (Fig. 1).

Western blot analysis of GR protein in HTC cells demonstrated, in addition to the major 94 kDa GR species, a minor 91 kDa protein only seen in untreated cells (Fig. 1). This 91 kDa protein was also seen in liver of adrenalectomized nonhormone treated rats (see below). The relationship of the 91 kDa protein to the 94 kDa GR is unknown.

Decay of GR mRNA and Protein in the Presence or Absence of Dexamethasone

GR mRNA stability in the presence or absence of dexamethasone was determined by analysis of GR mRNA decay after inhibition of transcription with actinomycin D. One set of HTC cells was treated with 0.5 μM dexamethasone for 24 h to cause down-regulation of GR mRNA levels. Actinomycin D was then added to these cells and to a parallel set of untreated cells whereafter the amount of GR mRNA was analyzed by RNA blot hybridization at various time points. The concentration of actinomycin D used (5 μg/ml) caused within 1 h an approximately 90% inhibition of ^{3}H-uridine incorporation into total cellular nucleic acids precipitable by trichloroacetic acid (data not shown). Figure 2 shows a summary of the results from densitometric scans of RNA blots. A half-life of GR mRNA of approximately 4.5 h was seen both in the presence or absence of dexamethasone. Thus, dexamethasone had no effects on the stability of GR mRNA. Neither did dexamethasone affect the stability of β-actin mRNA which was used as an internal control. The β-actin mRNA half-life was found to be approximately 12 h (not shown), similar to what has been described earlier (18).

The GR protein decay in HTC cells in the presence or absence of dexamethasone was determined immunochemically after inhibition of translation with cycloheximide. One set of cells was pretreated with 0.5 μM dexamethasone for 48 h to cause down-regulation of the GR protein (*c.f.* Fig. 1). After addition of cycloheximide to these cells and to a set of untreated cells, GR protein decay was analyzed by Western immunoblotting (Fig. 3). The concentration of cycloheximide used, 1.5 μg/ml, caused within 1 h approximately 95% inhibition of incorporation of ^{35}S-methionine into cytosolic protein precipitable by trichloroacetic acid (data not shown). Four hour after addition of cycloheximide, an approximately 25% increase in GR protein level was seen both in dexamethasone treated and untreated cells. After this initial increase, GR protein decayed in the absence of dexamethasone with first order kinetics with a half-life of approximately 25 h. In the presence of hormone, a biphasic decay was observed with 50% of the GR protein remaining after approximately 11 h. No change in actin protein half-life (first order kinetics) was seen after dexamethasone treatment (not shown).

Regulation of GR mRNA and Protein Levels in Rat Liver by Dexamethasone

The homologous regulation of GR gene expression was also studied in an animal model and compared to the data obtained with HTC cells. Adrenalectomized rats

were treated with a single dose of dexamethasone-1-phosphate (4 mg/kg) ip. Livers were removed at various time points after hormone administration and were analyzed for GR mRNA and protein levels. The amount of GR mRNA in rat liver decreased 4 h after administration of hormone (Fig. 4a). Maximal reduction (90%) was obtained after 18 h; at 48 h GR mRNA levels were partially restored. The same degree of changes was seen for the 6.5, 5.5 and 4.5 kilobase (kb) GR mRNA species. Three separate GR transcripts have also been described in human fibroblasts (19). The two larger RNA species most likely represent alternative processing products of the same transcription unit (20). The origin of the smallest RNA species is not known. Adrenalectomy 9 days before the experiment did not lead to any induction of GR mRNA levels in rat liver as compared to unoperated animals (data not shown). Prior studies using ligand binding techniques have shown that an approximately 1.5-fold increase in GR levels is seen 3–4 days after adrenalectomy followed by a return to essentially normal levels (21, 22, and Okret, S., unpublished results).

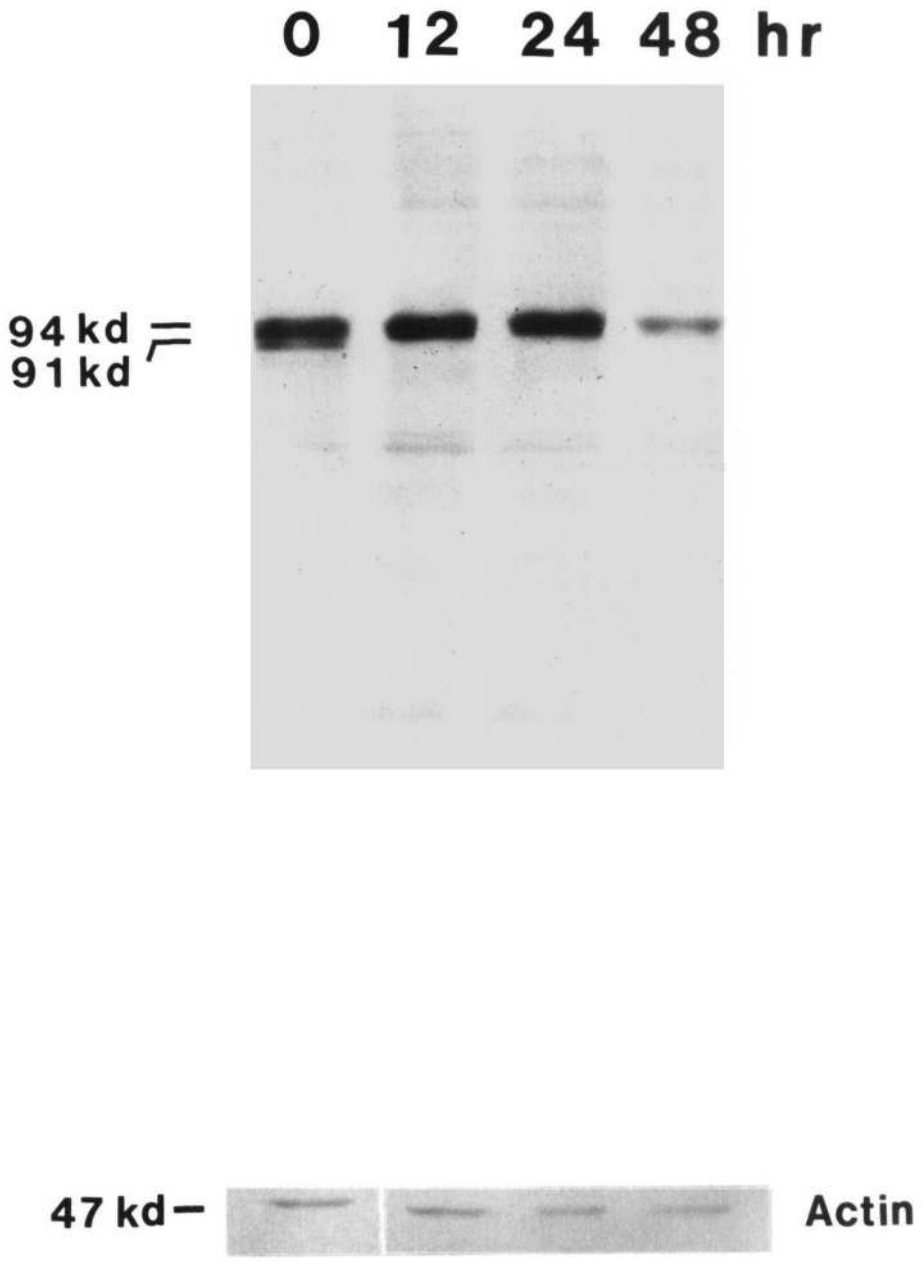

Fig. 1. Western Blot Analysis of GR and Actin Protein Levels in HTC Cells after Treatment with Dexamethasone

Cells were incubated with 0.5 μM dexamethasone for indicated times. After treatment, cellular extracts were prepared and subjected to Western blot analysis as described in *Materials and Methods*. Each lane contains 100 μg total cellular protein. The apparent sizes of the signals were estimated from the electrophoretic mobilities of standard proteins (phosphorylase b, 97.4 kDa; BSA, 67 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 30 kDa).

In parallel to determination of GR mRNA levels in rat liver, analysis of cellular GR protein content in the same livers was performed by Western immunoblotting (Fig. 4b). In contrast to GR mRNA, no down-regulation of GR protein was seen before 8 h or treatment with dexamethasone. The GR protein level reached its minimum value (80% reduction) 24 h after dexamethasone administration, *i.e.* with an approximately 8 h delay as compared to the GR mRNA decrease. The GR protein level was partially restored after 48 h. In contrast to the results obtained with HTC cells (Fig. 1), no initial up-regulation of either the GR mRNA or the GR protein level was seen in rat livers. As in the case with untreated HTC cells, livers from untreated adrenalectomized rats showed a minor immunogenic 91 kDa band in addition to the major 94 kDa GR protein. After administration of dexamethasone, the 91 kDa band disappeared (Fig. 4B). Both β-actin mRNA and actin immunoreactivity in the liver showed only minor changes due to the hormone treatment (Fig. 4, A and B).

Transcription Rate of the GR Gene in Rat Liver after Dexamethasone Administration

In order to determine whether the observed dexamethasone-induced down-regulation of the GR mRNA levels was due to a decreased transcription rate, nuclear run-on transcription experiments were performed using liver nuclei isolated from adrenalectomized rats treated with a single dose of dexamethasone-1-phosphate (4 mg/kg). Figure 5 shows a time course of the transcription rate of the GR gene after hormone treatment. Before 2 h, no decrease in GR gene transcription rate was seen. Within 2 to 4 h of treatment, GR gene transcriptional activity fell by about 50%. At 18 h, the transcription rate decreased to a minimum (75% reduction). At 24 h, the transcription rate had started return to normal. Thus, the dexamethasone-dependent changes in GR gene transcription rate paralleled the changes in GR mRNA levels.

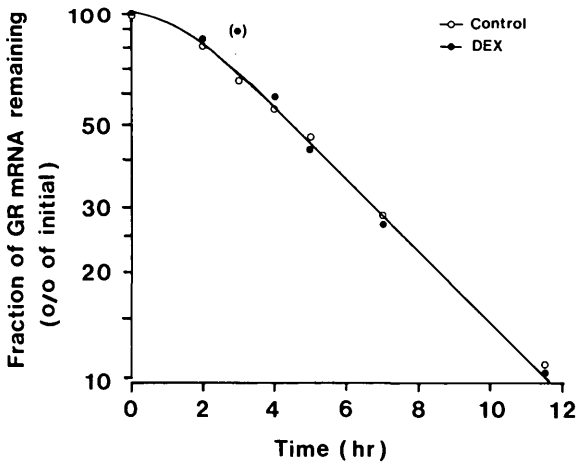

Fig. 2. Half-Life of GR mRNA in the Presence or Absence of Dexamethasone

HTC cells were grown in the presence (●) or absence (○) of 0.5 μM dexamethasone (DEX) for 24 h. Thereafter, 5 μg/ml actinomycin D were added to the cells for indicated times. The rate of GR mRNA decay was determined by measuring the GR mRNA levels by RNA blot hybridization (see *Materials and Methods*) at various time points after the addition of actinomycin D. The figure shows the mean values (in percent of the GR mRNA levels at the start time of actinomycin D treatment in the presence or absence of dexamethasone, respectively) from the results of two separate experiments. Relative quantification was performed by densitometric scanning of autoradiograms.

DISCUSSION

Steroid hormones modulate gene expression both positively and negatively (23). In most cases this effect has been shown to be due to a transcriptional effect after the interaction of the receptor with steroid-responsive elements of target genes (for reviews, see Refs. 1 and 2). However, posttranscriptional mechanisms alone or in combination with a transcriptional regulation may also be important determinants of steroid dependent gene expression. Brock and Shapiro (24) have shown that estrogens have a dramatic stabilizing effect on vitellogenin mRNA. Glucocorticoid hormones and triiodothyronine stimulate rat GH gene expression by an apparent combination of both transcriptional response and stabilizing effect on GH mRNA (18, 25). Glucocorticoids stabilize rat class I alcohol dehydrogenase mRNA (26). In contrast, glucocorticoids destabilize type I procollagen mRNA (27). Additional posttranscriptional, including posttranslational, mechanisms may be involved in steroid hormone controlled gene expression. In line with a posttranslational regulation, McIntyre and Samuels (16) have shown, using a dense amino acid labeling technique, that glucocorticoids affect the turnover of GR protein without affecting the rate of GR synthesis.

We and others (17, 19, 28) have previously shown that the glucocorticoid hormone mediated down-regulation of GR in cells or in tissues *in vivo*, determined by several investigators by ligand binding assay, is reflected at the mRNA level. We found, however, that regulation of GR mRNA in HTC cells is characterized by a cyclic pattern. In the present study, we have investigated the levels and mechanisms for the glucocorticoid mediated down-regulation of GR in HTC cells and in rat liver *in vivo*.

The changes in GR protein level in HTC cells treated with dexamethasone (Fig. 1) followed the changes in GR mRNA (*c.f.* Ref. 17), both with respect to the initial up-regulation and the following down-regulation, albeit with a 6- to 24-h delay. The significance of the up-regulation of GR mRNA and protein seen in HTC cells is presently not understood since the *in vivo* experiments with rat liver did not show any early increase in either GR mRNA or protein levels (Fig. 4). In order to establish that this particular pattern of changes in GR level in HTC cells were not due to effects of dexameth-

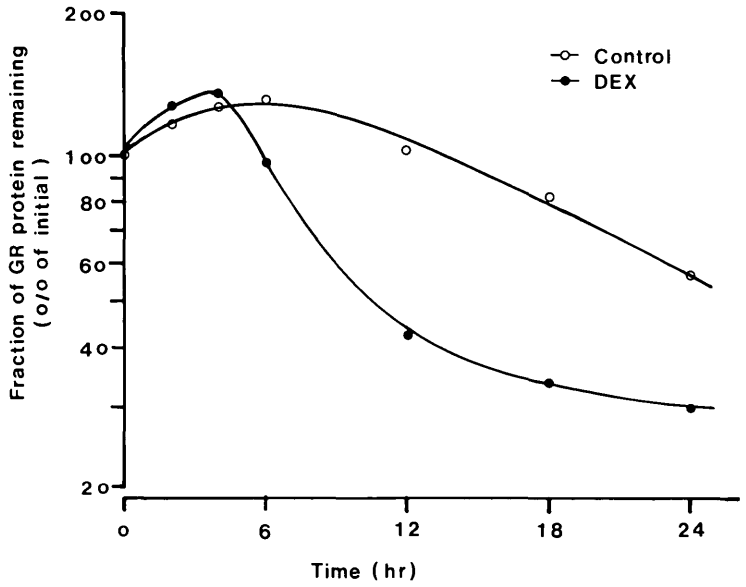

Fig. 3. Half-Life of GR Protein in the Presence or Absence of Dexamethasone

HTC cells were grown in the presence (●) or absence (○) of 0.5 μM dexamethasone (DEX) for 48 h. Thereafter, cells were exposed to 1.5 μg/ml cycloheximide. The rate of GR protein decay was determined by measuring total cellular GR protein levels by Western immunoblotting (see *Materials and Methods*) at various time points after the addition of cycloheximide. The figure summarizes the results from densitometric scans of two separate experiments. The data are presented in percent of the GR protein levels at the start time of cycloheximide treatment in the presence or absence of dexamethasone.

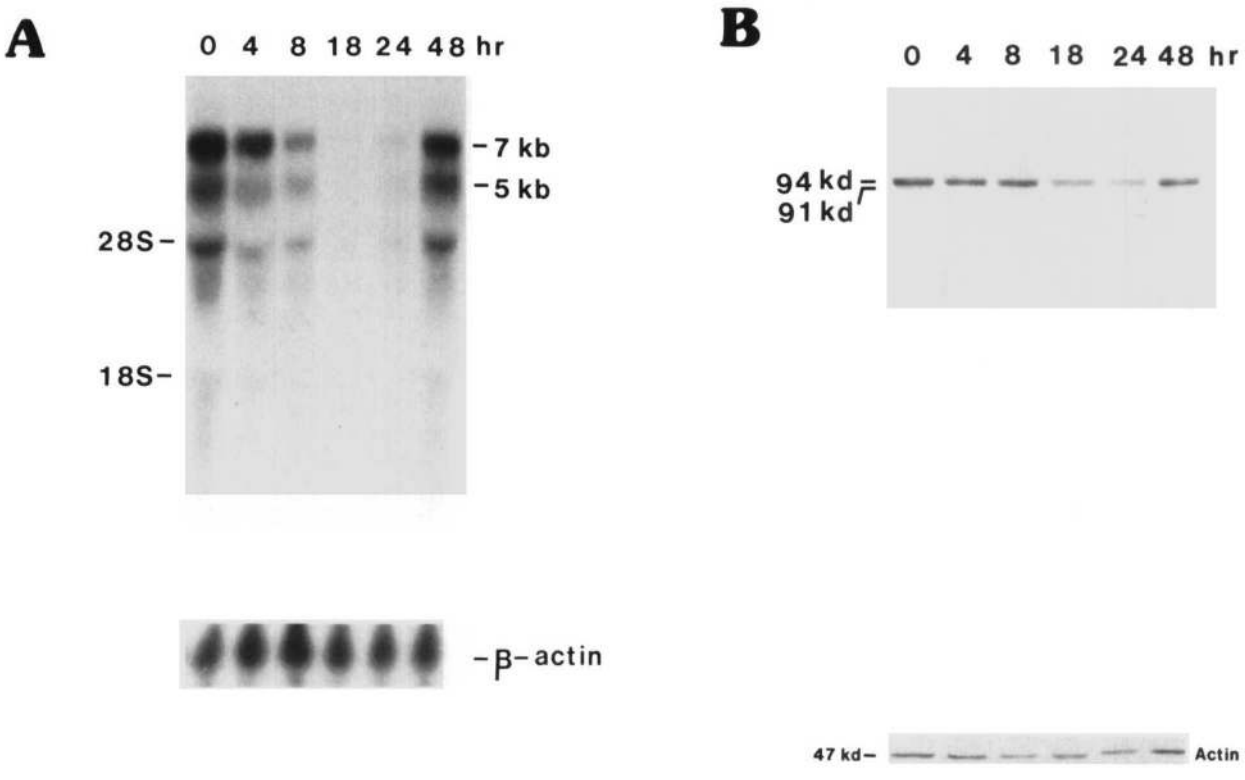

Fig. 4. The Analysis of GR mRNA and Protein Levels in Rat Liver after Treatment with Dexamethasone

Rats were adrenalectomized 9 days before treatment with dexamethasone-1-phosphate (4 mg/kg BW). At indicated times, total RNA and protein were isolated from the livers and analyzed for GR and β-actin mRNA by blot hybridization and for GR and actin proteins by Western immunoblotting (see *Materials and Methods*). A, Autoradiogram of RNA blots. The sizes of the signals are calculated from the positions of 18 S and 28 S rRNAs, respectively. B, Western blots. The sizes of the hybridization signals are determined from the mobility of standard proteins (see legend to Fig. 1).

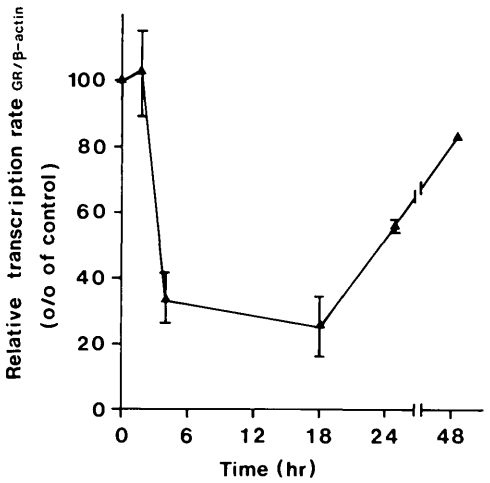

Fig. 5. GR Gene Transcription in Rat Liver after Treatment with Dexamethasone

Rats were adrenalectomized 9 days before treatment with dexamethasone-1-phosphate (4 mg/kg BW). After various times (0–48 h as indicated), cell nuclei were isolated from the livers and the nuclear run-on transcription experiments were performed as described in *Materials and Methods*. The radioactively labeled RNA retained on the filters was determined by scintillation counting. The specific hybridization of RNA to pRM16 and β-actin plasmid DNAs were determined by subtraction of nonspecific hybridization to pGEM3 and pBR322 vector DNAs, respectively. The relative transcription of GR mRNA is related to the transcription of β-actin mRNA and is expressed as the percentage of control values. Mean values and SD of results from three separate experiments are presented. For untreated animals, actual amount of RNA hybridized to the GR cDNA was around 200 cpm. RNA hybridized to β-actin cDNA was approximately 500 cpm. Background levels (pBR322 and pGEM3) were about 10–20 cpm.

asone on cell cycle phases, the growth pattern and thymidine incorporation of HTC cells were analyzed in the presence or absence of dexamethasone. Although dexamethasone (0.5 μM) inhibited HTC cell division for approximately 24 h, no synchronization of cell growth occurred (Dong, Y., and S. Okret, unpublished observations). This was important to exclude since it has been shown that cellular GR concentration varies during different cell cycle phases (29, 30).

Western blot analysis of GR from both cells and rat liver showed, in addition to the major immunogenic 94 kDa GR protein, a minor 91 kDa immunoreactive protein. This species was only seen in the absence of dexamethasone and its nature is obscure. However, we have previously demonstrated a proteolytic immunogenic fragment of the native 94 kDa GR protein with a mol wt of 91 K (31). Another possibility is that the 91 kDa species represents a GR variant resulting from an alternative site of initiation of translation on the GR mRNA (20).

The 6- to 24-h time lag in regulation of the GR protein level as compared to the GR mRNA seen both in HTC cells and rat liver may be due to the differences in half-lives of GR mRNA and GR protein, respectively. The half-life of GR mRNA was approximately 4.5 h, independently of whether dexamethasone was present or not (Fig. 2), indicating that glucocorticoids do not affect GR mRNA stability. In contrast, GR protein turnover rates were found to be approximately 2.5 fold faster in the presence than in the absence of dexamethasone (Fig. 3). In the presence of hormone a biphasic decay was seen. The mechanism behind this is unclear. The 6-h difference in GR mRNA and GR protein turnover rates in the presence of dexamethasone may explain why the GR protein level always responded somewhat more slowly to dexamethasone than the GR mRNA.

The initial 25% increase in GR protein level seen 4 h after addition of cycloheximide (Fig. 3) may be related to the approximately 3-fold superinduction of GR mRNA level caused by cycloheximide (17). If the superinductive effect of cycloheximide on GR mRNA level precedes the inhibitory effect of cycloheximide on translation, accumulation of GR mRNA may result in a small initial increase of the GR protein level as well.

Our immunochemically determined half-lives of GR protein in HTC cells after inhibition of protein synthesis with cycloheximide in the absence or presence of dexamethasone, agree well with the data obtained with GH cells by McIntyre and Samuels (16) using a dense amino acid labeling technique (16). They reported half-lives of 19 or 9.5 h for GR in the absence or presence of triamcinolone acetonide, respectively. Similar data were obtained from human IM-9 cells based on ligand binding assay (32). The increased receptor turnover in the presence of glucocorticoids could be due to that the GR translocated to cell nuclei is more susceptible to proteolytic breakdown than the cytoplasmic GR. Ligand induced activation of GR to a DNA binding state also has been suggested to involve a conformational change or a dissociation of subunits (33). In both models, it is conceivable that the susceptibility of GR with proteases might change after activation. Since glucocorticoid mediated down-regulation of GR protein, as well as GR mRNA (17), also occurs in the absence of protein synthesis, the increased receptor turnover cannot be due to increased synthesis of a GR modulating protein (14, 16, 32).

To summarize, down-regulation of GR mRNA is caused by a decreased transcription rate of the GR gene. However, GR protein autoregulation is complex since it also involves posttranslational regulation as apparent from the faster turnover rate of GR in the presence of dexamethasone. In case transcriptional as well as posttranslational mechanisms are involved in regulating GR expression, down-regulation of GR protein should exceed down-regulation of GR mRNA. In contrast, we observed in rat liver that down-regulation of GR mRNA exceeded down-regulation of GR protein (Northern and Western analysis performed on different pieces of the same livers). This could indicate that other control mechanisms neutralizing the changes in GR mRNA levels may exist. In line with such a possibility, McIntyre and Samuels (16) have shown in pituitary cells, that glucocorticoids do not affect the synthesis rate of

GR. It has also recently been speculated that a possible covalent modification of down-regulated TRH receptor mRNA causes a decreased posttranscriptional activity of the mRNA (34). For some other transcripts, hormones are known to affect translation efficiencies (35, 36).

During preparation of this manuscript, Rosewicz *et al.* (37) presented data from a different cell line, consistent with our observation, that the down-regulation of GR mRNA by glucocorticoids was due to decreased glucocorticoid receptor gene transcription. However, no translational or posttranslational data were presented.

Autologous negative feed back by the cognate ligand on its own receptor mRNA has also been demonstrated for the low density lipoprotein receptor (38) and, as mentioned above, the TRH receptor (34). The synthetic estrogen, diethylstilbestrol, down-regulates the estrogen receptor mRNA level (39). Progestins down-regulate progesterone mRNA (40). The transferrin receptor mRNA is down-regulated by iron or hemin (41). An autologous positive feed back by the cognate ligand on its own receptor mRNA has been shown for the 1,25 dihydroxyvitamin D_3 (42), epidermal growth factor (43), and interleukin 2 (44).

The transcriptional response of the GR gene to dexamethasone as well as the observed down-regulation of GR mRNA in the absence of protein synthesis suggest that autoregulation of GR may occur via the interaction of GR with its own gene. In line with this, we have previously demonstrated that GR *in vitro* specifically interacts with a GR cDNA fragment corresponding to the 3′-untranslated region of GR mRNA (17). We are currently investigating whether this fragment is of significance *in vivo* with regard to GR regulation.

MATERIALS AND METHODS

Cell Culture and Animals

Rat Reuber hepatoma cells (H4IIE, hepatoma tumor cell line HTC) were obtained from the American Tissue Culture Collection and grown in RPMI1640 (GIBCO, Grand Island, NY) medium supplemented with 8% (vol/vol) heat-inactivated and charcoal-treated (1 h, 37 C) fetal calf serum (GIBCO), 2 mM L-glutamine (Flow Laboratories, McLean, VA) benzylpenicillin (400 IU/ml; Astra, Södertälje, Sweden), and streptomycin (0.2 mg/ml; Novo, Copenhagen, Denmark). When indicated, cells were treated with 0.5 μM dexamethasone (Sigma, St. Louis, MO; dissolved in ethanol), 1.5 μg/ml cycloheximide or 5 μg/ml actinomycin D (Sigma, dissolved in water). Control cells were treated with solvent only. Ethanol concentration was kept below 0.005%. Cell viability as determined by trypan blue exclusion was greater than 90% for all conditions of treatment during the experimental time periods.

Male Sprague-Dawley rats weighing 200–250 g were used. Adrenalectomy was performed under anesthesia (4 mg fluarison, 0.12 mg fentamyl-dihydrogencitrate, and 4 mg diazepam/kg BW). Dexamethasone-1-phosphate (Merck Sharp and Dohme, Haarlem, The Netherlands) administration was carried out by ip injections. Control animals were injected with PBS. Adrenalectomized rats received food *ad libitum* and water containing 0.5% (wt/vol) NaCl. Animals were kept in a 12-h light and dark cycle and killed by cervical dislocation.

Recombinant Clones

Plasmid pRM16 that carries a 2.6 kilobasepairs cDNA insert corresponding to the 3′-untranslated end of the rat GR mRNA (45), was cloned into the transcription vector pGEM3 (Promega Biotec), and used in the nuclear run-on transcription experiments. A 700 basepairs (bp) EcoRI-PstI rat GR cDNA fragment corresponding to the 3′ portion of the coding region was isolated from pRM9 (20) and cloned into the pSP65 vector (Promega Biotec, Madison, WI) to produce p9:700 and a cRNA probe by SP6 polymerase for use in the RNA blot hybridization experiments. A cDNA probe for β-actin cloned into pBR322 was from Cleveland *et al.* (46). pBR322 plasmid DNA was obtained from Boehringer Mannheim (Indianapolis, IN).

Isolation of RNA and Blot Hybridization

Total RNA from HTC cells was prepared by homogenization of cells in 4 M guanidinium thiocyanate and centrifugation of the homogenate through a cushion of 5.7 M CsCl (47). RNA from rat liver was isolated according to a single-step method described by Chomczynski and Sacchi (48). Twenty micrograms of total RNA were separated on 0.9% (wt/vol) agarose-formaldehyde gels and blotted onto nitrocellulose filters by capillary force (49). Filters were baked under vacuum for 2 h at 80 C and hybridized with 3×10^6 cpm p9:700 ^{32}P-cRNA probe/ml hybridization buffer (50) for 16–20 h at 60 C. Washing was carried out in 30 mM NaCl/3 mM sodium citrate, pH 7.0, 0.1% sodium dodecyl sulfate buffer, four times for 5 min at room temperature, and then twice for 1 h at 65 C. The β-actin ^{32}P-cDNA probe was prepared by nick translation (50) and hybridized with RNA filter at 50 C and washed at 55 C in the same buffer as above. The autoradiograms were analyzed densitometrically with a Shimadzu Dual-Wavelength TLC scanner CS-930 (Kyoto, Japan). Quantitative analysis of autoradiograms were always performed on underexposed films. These estimations correlate well with counts per min values obtained by liquid scintillation counting of the specific bands. Hybridization of the Northern filters with labelled GR mRNA gave no signal (not shown).

Preparation of Cellular Extract and Western Immunoblotting

Cells or tissues were lysed and homogenized in TEG buffer (10 mM TrispHCl, pH 7.4, 1 mM EDTA, 10% (vol/vol) glycerol and 2 mM dithiothreitol) containing 0.4 M NaCl. Under these conditions, greater than or equal to 90% of the hormone-GR complexes in the cell nuclei were extracted. After centrifugation of the homogenate at 200,000 × *g* for 45 min at 4 C, the supernatant was precipitated by ammonium sulfate (0.2 g/ml). The pellet was dissolved in TEG buffer without salt. Protein concentration was determined according to Lowry *et al.* (51) and by measurement of optical density at 280-310 nm. One hundred micrograms of protein per lane were separated on a 7% sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto nitrocellulose filter (Bio-Rad protocol, Richmond, CA). The GR protein bands were identified by the monoclonal anti-GR antibodies (no. 8, Ref. 52) and visualized by the Proto Blot Immunoblotting System (Promega Biotec) using an alkaline phosphatase conjugated second goat anti-mouse antibody. The signal intensity of the filters was determined by measuring reflections using a densitometer as described above. The GR signal intensity analyzed were always in the linear range of a standard curve using different amounts of GR protein. Western blots were also analyzed for actin protein by a monoclonal mouse anti-chicken actin antibody (BioGenex Laboratories, Dublin, CA) as an internal control of the amount of protein applied.

Nuclear Run-On Transcription Analysis

Cell nuclei from rat liver were purified according to Blobel and Potter (53). Briefly, excised rat liver was immediately homog-

enized in 0.25 M sucrose solution (containing 50 mM Tris-HCl, pH 7.5, 25 mM KCl, and 5 mM $MgCl_2$) and mixed with 2 vol 2.3 M sucrose in the same buffer. The final homogenate was then layered on a cushion of 2.3 M sucrose solution and centrifuged at 200,000 × *g* for 1 h at 4 C. The resulting nuclear pellet was washed with TGEM buffer (50 mM Tris-HCl, pH 8.3, 40% (wt/vol) glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA) and stored at −70 C. The transcription analysis was carried out essentially as described (54). Nuclei corresponding to 100 μg DNA were resuspended in 50 μl 1.4 × TGEM buffer and mixed with 50 μl transcription reaction mixture (0.5 mM each ATP, CTP, and GTP, 20 μM UTP in buffer-10 mM Trisp-HCl, pH 8.0, 5 mM $MgCl_2$, and 300 mM KCl) and 200 μCi (α-^{32}P)UTP (400 Ci/mmol, Amersham, England). Reaction mixtures were incubated for 30 min at 25 C and then terminated by the addition of 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM KCl, 1 mM Na_2EDTA and 0.5% sodium dodecyl sulfate buffer. Incorporation of ^{32}*P*-UTP into total RNA was linear up to 30 min. RNA was extracted from nuclei and purified as described (55). The *in vitro* labeled transcripts were hybridized with nitrocellulose filters for 72 h at 42 C (10 × 10^6 cpm/filter). Each filter contained 5 μg linearized plasmid DNA pRM16, pGEM3, β-actin, and pBR322 immobilized as described (50). The washing conditions were the same as described above for Northern blotting. The amount of hybridized RNA was determined by scintillation counting.

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