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THE MECHANISM FOR GLUCOCORTICOID-RESISTANCE IN A RAT HEPATOMA CELL VARIANT THAT CONTAINS FUNCTIONAL GLUCOCORTICOID RECEPTOR

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Summary—The mechanism of glucocorticoid resistance has been studied in a rat hepatoma cell variant (6.10.2), which contains low levels of glucocorticoid receptor (GR). These cells seem to have lost all the glucocorticoid-induced transcriptional responses as measured by the lack of induction of expression of stably integrated mouse mammary tumor virus (MMTV) and the endogenous gene tyrosine aminotransferase (TAT), as well as the transcriptional suppression of GR gene expression. Physico-chemical characterization of the GR in the glucocorticoid resistant 6.10.2 cells revealed that the receptor is indistinguishable from the wild-type receptor with regard to size, hormone- and DNA-binding. The levels of the receptor mRNA and the total immunoreactive protein found in 6.10.2 cells were about 20% of those found in wild-type cells. Further analysis of 6.10.2 cells demonstrated that the receptor was indeed biologically functional. Treatment of 6.10.2 cells with 8-bromo-cAMP, which induced the endogenous GR level two-fold, restored responsiveness to glucocorticoids. Secondly, pretreatment of the cells with cycloheximide also led to reacquisition of cellular responsiveness to glucocorticoids. We propose that there exists a "threshold" level of GR, which is required for responsiveness and that under normal culture conditions, the level of GR in 6.10.2 cells is below this threshold. Glucocorticoid responsiveness can be restored by raising the GR level above the threshold with 8-bromo-cAMP or, alternatively, by removing the threshold barrier (repressor protein) with cycloheximide. Finally, the existence of such a repressor protein for MMTV induction was shown by *in vivo* titration with an isolated negative *cis*-element from the MMTV promoter.

INTRODUCTION

A prerequisite for biological effects following glucocorticoid administration is the presence of a functional glucocorticoid receptor (GR) protein in the cell. The ligand activated GR–hormone complex mediates its effects by binding to glucocorticoid responsive elements (GREs) in target genes. This leads to the modulation of expression of a network of genes by coordinately inducing or repressing the transcriptional activity in a gene and tissue specific way (for a review see Ref. [1]). Molecular and functional analysis of purified and cloned GR have revealed that the GR consists of at least three independent functional domains; a carboxy-terminal steroid-binding domain, a highly conserved central DNA-binding domain and an amino-terminal domain which is essential for full transactivating activity [2–6]. Mutations in any of these domains can result in loss of glucocorticoid responsiveness.

A number of variants of glucocorticoid resistant cell lines have been isolated by genetic selection and their GR characterized. In most cases glucocorticoid resistance correlated to the loss of GR or to a change in GR structure with impaired functional activity (for a review see Ref. [7]). These mutations include e.g. loss of hormone–binding ability, decreased nuclear translocation and DNA-binding activity, deletion of the amino-terminal domain of the GR protein or activation labile receptor. In several cases, detailed molecular analysis has identified the precise mutation giving rise to the particular receptor phenotype [8, 9]. However, examples of resistance despite the presence of functional receptors also exist [10]. Furthermore, one particular cell line which contains low levels of GR, was found to be nonresponsive by one parameter but responsive by another [11]. This may

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Abbreviations: GR, glucocorticoid receptor; MMTV-LTR, mouse mammary tumor virus long terminal repeat; TAT, tyrosine aminotransferase; GRE, glucocorticoid responsive element.

indicate the importance of reaching a threshold level in GR concentration to obtain glucocorticoid responsiveness which may vary for different target genes [12, see also Discussion]. Passing this threshold level in GR concentration, most data indicate a strong correlation between GR level and the magnitude of biological response [13, 16].

We report here the characterization of the GR protein from a previously described glucocorticoid nonresponsive hepatoma cell line, 6.10.2 [15, 17, 18]. This cell line contains low levels of GR and previous studies have shown that glucocorticoid treatment failed to induce endogenous tyrosine amino transferase (TAT) activity or stably integrated mouse mammary tumor virus (MMTV) mRNA. The defect could, however, be complemented by expression of wild type receptor cDNA [15, 18]. We find that the GR in 6.10.2 cells is indistinguishable from the wild-type protein. We have previously shown that the level of GR can be induced by 8-bromo-cAMP or forskolin resulting in an increased responsiveness to glucocorticoids [16]. We show here that it is possible to overcome the nonresponsiveness in 6.10.2 cells when the level of GR is elevated by 8-bromo-cAMP. This shows that the cells still contain functional GR. It was also possible to overcome the nonresponsiveness in this cell line by treating the cells with cycloheximide or transfecting the cells with a DNA-element from the MMTV promoter thought to interact with a repressor protein. The implications of these observations are outlined in the Discussion.

EXPERIMENTAL

Cell culture

M1.19 and M1.54.762 (762) are cloned lines of stably MMTV-infected rat hepatoma tissue culture cells. Transcription of MMTV is induced in these cells [19]. NSM6.10 is a clonal derivative of M1.19, selected for its low expression of viral glycoprotein gp52 in the presence of dexamethasone by fluorescence-activated cell-sorting [17] and has subsequently been shown to contain low levels of glucocorticoid receptor. 6.10.2 is an unselected subclone of NSM6.10 isolated in K. R. Yamamoto's laboratory [18].

Cells were grown in monolayer and cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 8% (v/v) heat-inactivated fetal calf serum (Gibco) and bensylpenicillin/streptomycin (Gibco). Serum steroids were stripped by dextran-coated charcoal treatment (1 h, 37°C).

Preparation of cytosol and crude cellular extracts

Either fresh or frozen cells were homogenized in ETG buffer (1 mM EDTA, 10 mM Tris–HCl, pH 7.8, 10% glycerol and 2 mM dithiothreitol). The homogenates were centrifuged at 96,000 *g* for 45 min at 4°C and the resulting supernatant was used as cytosol. For Western immunoblotting assays, crude cellular extracts were prepared as described above except that cells were homogenized in ETG plus 0.4 M NaCl.

Hormone binding analysis

Cytosols of both 762 and 6.10.2 cells were incubated with different concentrations (0.25–100 nM) of [1,2,4,6,7-^{3}H]dexamethasone (94 Ci/mmol, Amersham, Bucks., England) alone or in the presence of a 100-fold excess of unlabeled dexamethasone (Sigma) at 4°C for 18 h in a volume of 200 μl. Aliquots were taken from the incubation mixtures for determination of total input of radioactivity. Totally and non-specifically bound [^{3}H]dexamethasone were measured by hydroxylapatite (HAP) adsorption assay. 200 μl of a 50% (v/v) HAP slurry in ETG was added to the binding mixtures and incubated for 15 min under rotation. HAP adsorbed proteins were pelleted and washed 5 times with 1 ml ETG buffer containing 50 mM potassium phosphate. Proteins were then eluted from the resin with 200 μl 0.5 M potassium phosphate buffer, pH 7.8. Specific binding was quantitated by Scatchard analysis [20].

Western immunoblotting

Crude cellular extracts were concentrated by ammonium sulfate precipitation (0.25 g of solid ammonium sulfate per ml extracts). Protein concentration was determined according to Bradford [21]. The identification of GR by Western immunoblotting using a monoclonal anti-GR antibody was carried out as described [22] except that sheep anti-mouse [^{125}I]immunoglobulin (Amersham) was used as the second antibody. Filters were autoradiographed and the immunoreactive bands were quantitated by gamma-counting.

Isolation of RNA and blot-hybridization

Total cellular RNA was isolated according to Chomzynski and Sacchi [23]. RNA blots and

hybridization with radio-labeled probes were carried out as described [22]. Recombinant plasmids used for probe labeling have been described [16]. The autoradiograms of RNA blots were analyzed densitometrically with a Shimadzu dual-wavelength TLC scanner, cs-930 (Kyoto, Japan). Alternatively, the specific bands were excised and radioactivity determined by scintillation counting.

Assay of tyrosine aminotransferase activity

The assay was carried out according to the method of Diamondstone [24].

DNA–cellulose chromatography

DNA–cellulose (4 mg double stranded DNA per g resin) was obtained from Sigma (St Louis, MO, U.S.A.). A 2 ml DNA–cellulose column was prepared in ETG buffer. Cytosols were labeled with 100 nM [6,7-^{3}H]triamcinolone acetonide (New England Nuclear, Boston, MA, U.S.A.) at 4°C for 1 h. Receptor activation was carried out by heat-treatment at 25°C for 30 min. 1 ml cytosol was applied to the column, washed with ETG buffer and eluted with a linear (0–0.5 M) NaCl-gradient in ETG buffer.

Gel retardation assay

Cytosol was labeled with 100 nM dexamethasone and concentrated by ammonium sulfate precipitation. Protein was resuspended in ETG buffer at a concentration of 10–20 mg/ml. 0.2 ng (20,000 cpm) of [^{32}P]labeled GRE (see below) probe and 50 μg cytosolic protein were incubated in a 20 μl binding reaction. The binding buffer was the same as described [25]. When indicated, various amounts of radioinert GRE or nonspecific competitor DNA (see below) were included in the reaction mixture. After 15 min at 25°C, samples were loaded onto a 4% polyacrylamide gel and electrophoresed. The gels were dried and autoradiographed. Specific bands were excised and quantitated by ligand scintillation counting.

The synthetic oligonucleotide GRE was from the promoter region of the rat TAT gene [26] and the nonspecific competitor DNA (NC) was a synthetic oligonucleotide encompassing a nonrelated sequence. The upper strand sequences of both GRE and NC are shown below:

GRE: 5′GACCCTAGAGGATCTGTACAG

GATGTTCTAGATCCAATTCG-3′

NC: 5′GGATCCACCCTGTCTCATGAA

TATGCAAATCAGGTGAG-3′

Plasmids and transfection

A synthetic oligonucleotide corresponding to position −147 to −163 in the MMTV promoter was subcloned into the XbaI site of pGEM3 (Promega). 10 μg of this plasmid, pRRE1, or pGEM3 was transfected to 6.10.2 cells grown in 100 mm dishes to near confluency by the lipofectin method [27]. 30 μg of lipofectin was used per dish. The cells were incubated for 7 h, washed with DMEM containing 10% fetal calf serum and followed by an additional incubation period for 18 h in the presence or absence of 0.25 μM dexamethasone. RNA was processed and analyzed as described above.

RESULTS

Regulation of GR by dexamethasone in 6.10.2 cells

Classification of 6.10.2 cells as glucocorticoid unresponsive was based on the observation that treatment of the cells with dexamethasone failed to induce transcription of several target genes, e.g. those of MMTV, TAT and α-1-acid glycoprotein [15, 18]. Glucocorticoids not only stimulate but also suppress gene expression [1]. Since the mechanism(s) whereby the glucocorticoid–receptor complex may both activate or inhibit gene expression is still poorly understood, we considered it important to examine 6.10.2 cells with regard to negative regulation of genes by glucocorticoids. An example of a gene which is transcriptionally down-regulated by glucocorticoids is the GR gene itself [22, 28].

The glucocorticoid resistant 6.10.2 cells were treated with 0.5 μM dexamethasone for 24 h. The levels of GR mRNA were analyzed using an RNA blot hybridization technique. As shown in Fig. 1, treatment of cells with dexamethasone had no effect on the level of GR mRNA in contrast to the situation with the wild-type hepatoma cell line 762 where GR mRNA was down-regulated (cf. above). Thus, also a negatively glucocorticoid regulated gene fails to respond in 6.10.2 cells.

Characterization of GR in 6.10.2 cells

A functionally active GR is a prerequisite for glucocorticoid induced modulation of gene transcription. It is known that under normal culture conditions, 6.10.2 cells contain a lower

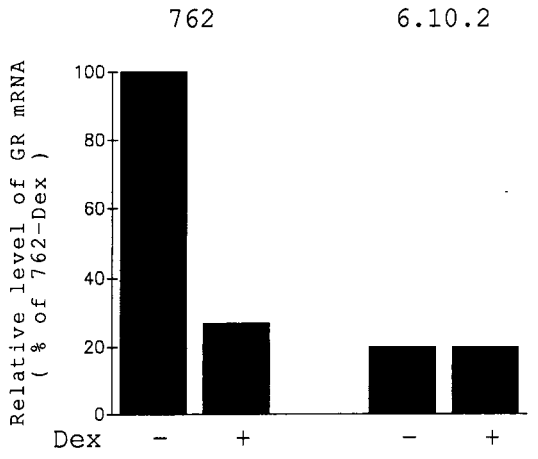

Fig. 1. Effect of dexamethasone on the levels of GR mRNA in 762 and 6.10.2 cells. Cells were grown in the presence or absence of 0.5 μM dexamethasone (Dex) for 24 h. The GR mRNA was analyzed by an RNA slot-blot-hybridization assay using a ^{32}P-labeled GR cRNA as probe (see Experimental). The data are summarized from four experiments.

glucocorticoid binding capacity as compared to wild-type cells [15, 18]. However, it is not known whether the low GR concentration in itself or a mutational defect in the GR protein is the cause for the resistant phenotype in 6.10.2 cells. We carried out experiments to characterize GR in 6.10.2 cells and compared the data with wild-type receptor in glucocorticoid-sensitive 762 cells.

Table 1 summarizes data obtained from both 762 and 6.10.2 cells. On the Northern blot, two transcripts, 7 and 5 kb, characteristic of the rat GR, were detected in both cell lines. The amount of GR mRNA expressed in 6.10.2 cells was around 20% of the level detected in 762 cells as measured by densitometric scanning of the autoradiograms. On the Western immunoblot, a monoclonal anti-GR antibody reacted with a 94 kDa component in both cell lines. Quantitation of the ^{125}I-labeled immunoreactive bands revealed that 6.10.2 cells contained about 20% immunoreactive GR as compared to 762 cells.

To determine the hormone-binding activity, cytosol preparations of cells were incubated with [^{3}H]dexamethasone in the presence or absence of an excess of unlabeled hormone. The maximum number of binding sites (B_{max}) and the dissociation constant (K_d) were calculated according to Scatchard [20]. It was found that B_{max} of 6.10.2 cells was about 14–20% of that found in 762 cells; i.e., 21–30 vs 145–150 fmol per mg protein (Table 1). However, the K_d values of the receptor–ligand complex were found to be similar in both cell lines (0.7–1.6 nM in 6.10.2 cells and 1–3 nM in 762 cells, Table 1).

Affinity of the GR for nonspecific DNA was assessed by DNA–cellulose chromatography. Cytosols prepared from both 762 and 6.10.2 cells were labeled with [^{3}H]triamcinolone acetonide, heat activated and analyzed on DNA–cellulose columns. Proteins were eluted with a linear salt gradient. The GR from 6.10.2 cells was eluted from the column at 185 mM NaCl which was virtually identical to the salt concentration required to elute the GR from 762 cells (180 mM, Table 1).

To study the binding properties of the GR to a specific DNA sequence, we utilized a gel retardation assay. In this assay, a synthetic oligonucleotide containing a functional GRE sequence derived from the TAT promoter was used as the probe for specific GR–DNA interaction. Cytosolic GR labeled with dexamethasone was heat activated and incubated with [^{32}P]GRE probe in the presence or absence of unlabeled competitor DNA. As shown in Fig. 2A, three retarded bands (1, 2 and 3) were formed when cytosols from both 762 and 6.10.2 cells were analyzed. Only formation of complex 1 was competed for by an excess of unlabeled GRE. However, nonrelated DNA did not compete for the formation of this complex indicating that complex 1 was the result of a specific interaction between GR and GRE. Figure 2B summarizes the data of competition experiments. Competition with increasing amounts of unlabeled GRE showed that the GR from both 762 and 6.10.2 cells bound to GRE with very similar affinity.

6.10.2 cells contain functional GR—recovery of cellular glucocorticoid sensitivity

Physicochemical characterization of GR from 6.10.2 cells did not reveal any major defects in important functions such as hormone and DNA

Table 1. Characterization of GR from glucocorticoid sensitive (762) and glucocorticoid resistant (6.10.2) cells

	[^{3}H]dexamethasone		DNA–cellulose	Size	
Cell line	K_d (nM)	B_{max} (fmol/mg)	NaCl (mM)	Protein (relative abundancy)	mRNA (relative abundancy)
762	1–3	145–150	180	94 kDa (100%)	7 and 5 kb (100%)
6.10.2	0.7–1.6	21–30	185	94 kDa (20%)	7 and 5 kb (20%)

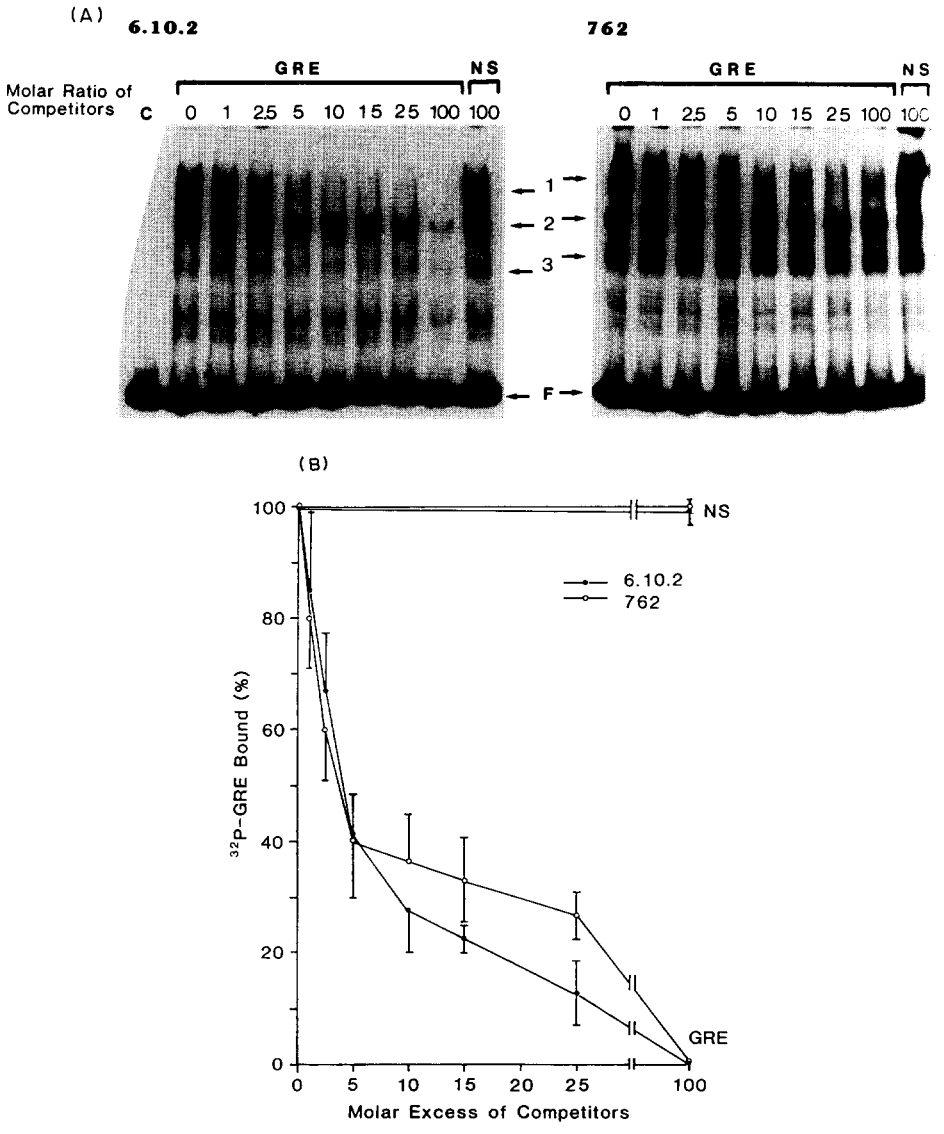

Fig. 2. Specific GR–DNA interaction studied by gel retardation assay. Dexamethasone labeled cytosolic proteins were incubated with ^{32}P-labeled GRE probe with or without various molar ratio excess of radio-inert competitor DNAs. The samples were subsequently electrophoresed through a nondenaturing polyacrylamide gel. (A) Autoradiograms of the gels. NS, nonspecific competitor DNA; C, sample without cytosolic protein; F, free probe; 1, 2 and 3, retarded protein–DNA complexes. (B) Quantification of radioactivity retained in complex 1. The data were summarized from four separate experiments and presented as molar excess competitor DNA versus the amount of complex 1 and expressed as a percentage of complex 1 in the absence of competitor. The radioactivity in the samples with 100 molar excess GRE was used as the background value and subtracted from each sample.

binding, although minor mutations in the receptor could still not be excluded. However, the concentration of GR in 6.10.2 cells is low which possibly might determine glucocorticoid insensitivity. Thus, if the endogenous receptor level was increased, it might be possible to override the glucocorticoid unresponsiveness of 6.10.2 cells. Recently, we have shown that cAMP treatment of rat hepatoma cells (both H411E and 762) increases both the cellular amount of GR protein and glucocorticoid responsiveness [16]. 6.10.2 cells were treated in a similar way with 50 μM 8-bromo-cAMP in the presence or absence of 0.5 μM dexamethasone for 18 h and analyzed for the expression of GR, MMTV and TAT. In the case of expression of the GR mRNA, dexamethasone alone had no effect (Fig. 3). Treatment of the cells with 8-bromo-cAMP induced the GR mRNA level about two-fold (lane 3 in Fig. 3) consistent with the result obtained in wild-type cells [16]. This increase in GR mRNA level by 8-bromo-cAMP was reflected in a similar degree of increase in the GR protein level (data not shown). Treat-

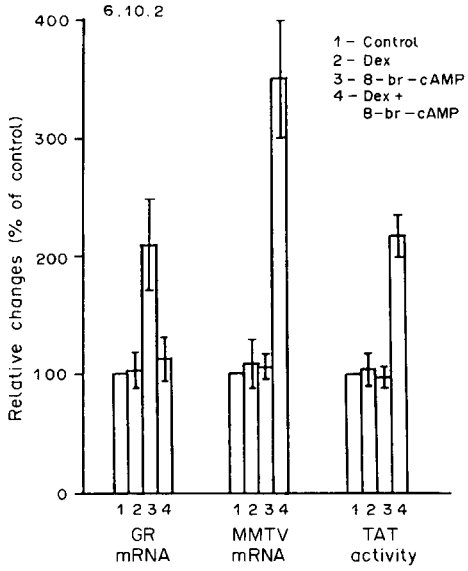

Fig. 3. Effect of 8-bromo-cAMP on dexamethasone mediated cellular responses in 6.10.2 cells. Cells were pretreated with 50 μM 8-bromo-cAMP(8-br-cAMP) for 4 h and then exposed to 0.5 μM dexamethasone (Dex) for 18 h. Total cellular RNA was isolated and analyzed for GR mRNA and MMTV mRNA (see legend to Fig. 1). For the studies of the enzyme activity of TAT, cells were treated in a similar way and the enzyme assay was performed as described in Experimental. The level of each sample was related to the control which was set to be 100%. The data are summarized from four experiments.

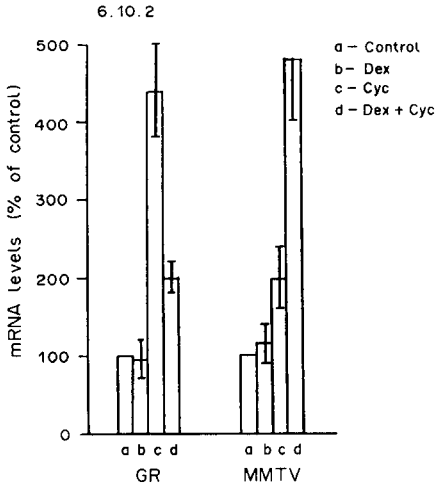

Fig. 4. Effect of cycloheximide on dexamethasone induced changes of mRNA levels in 6.10.2 cells. 6.10.2 cells were treated with 0.5 μM dexamethasone (Dex) in the presence or absence of 1.5 μg/ml cycloheximide (Cyc) for 18 h. The concentration of cycloheximide used inhibited protein synthesis about 95–99% [22]. Total cellular RNA was extracted and analyzed for the levels of mRNA of both GR and MMTV. The level of mRNA in each sample was related to the mRNA level in control cells. The data are summarized from four experiments.

ment of cells with 8-bromo-cAMP restored negative glucocorticoid regulation of gene expression since GR mRNA was now down-regulated by dexamethasone (compare lines 3 and 4 in Fig. 3). In the case of the expression of MMTV mRNA and the enzyme activity of TAT, neither dexamethasone nor 8-bromo-cAMP alone had any effect (Fig. 3, lanes 2 and 3). However, combined treatment of cells with both agents significantly induced the level of MMTV mRNA as well as the activity of TAT (Fig. 3, lane 4). The above results demonstrate that the two-fold increase in the endogenous GR concentration at least partly restored hormone sensitivity in 6.10.2 cells.

We have previously shown that rat hepatoma cells treated with the protein synthesis inhibitor cycloheximide, exhibited a higher steady-state level of the GR mRNA [29]. One of the possible explanations was that cycloheximide inhibited the synthesis of a rapidly turned-over inhibitory *trans*-acting factor, i.e. a repressor, and consequently increased the rate of transcription of the GR gene.

To test the possibility that a repressor protein suppresses basal level expression of the GR gene in 6.10.2 cells, the cells were treated with 1.5 μg/ml cycloheximide in the presence or absence of 0.5 μM dexamethasone for 18 h. Cellular levels of the GR mRNA were analyzed by RNA blot hybridization. For comparison, the level of MMTV mRNA was also analyzed simultaneously. Cycloheximide increased the GR mRNA level about 4.4-fold (Fig. 4, lane C). The effect of cycloheximide on the mRNA level of MMTV was not as pronounced as for GR mRNA, but a significant 2-fold increase was obtained (Fig. 4, lane C). Dexamethasone alone had no effect on either GR mRNA or MMTV mRNA (Fig. 4, lane B). Surprisingly, in the presence of cycloheximide, both the expression of GR and MMTV mRNAs were modulated by dexamethasone; the GR mRNA level was down-regulated by 60% (compare lanes C and D in Fig. 4) and the MMTV mRNA was increased by a factor of about 2.5-fold (compare lanes C and D in Fig. 4). The above results demonstrate that 6.10.2 cells regained certain hormone regulated responses after cycloheximide treatment.

Some hormone regulated genes are known to contain *cis*-elements that bind nuclear factors and exert a negative effect on gene expression [30, 31]. Also the MMTV-LTR has been shown to contain negative regulatory sequences influencing promoter activity [32, 33]. Such a negative element is found in the MMTV-LTR between −147 and −163 relative to the

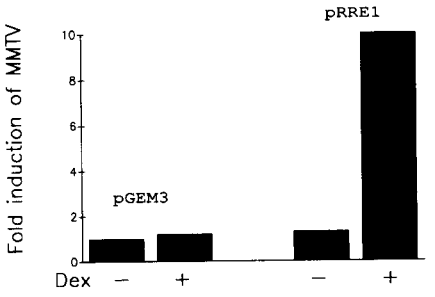

Fig. 5. MMTV induction following *in vivo* titration by transfecting 6.10.2 cells with a plasmid containing a putative repressor binding site. Cells were grown to near confluency and transfected with 10 μg of pRRE1 or pGEM3 as control. After treatment with 0.25 μM dexamethasone for 18 h, total RNA was isolated and analyzed by RNA blot-hybridization with a labeled MMTV probe (see Materials and Methods). Levels were normalized to β-actin expression. Data are summarized from three separate experiments.

start site of transcription [32]. We were interested to see whether this element, here called RRE, may repress glucocorticoid mediated induction of MMTV expression in 6.10.2 cells. We therefore performed an *in vivo* competition assay, transfecting 6.10.2 cells with a vector containing an RRE oligonucleotide (pRRE1). Titration of a factor interacting with RRE resulted in the restoration in 6.10.2 cells of glucocorticoid inducible expression of the stably integrated MMTV (7.7-fold induction, see Fig. 5). A vector lacking the RRE oligonucleotide (pGEM3), did not restore inducibility. Gel retardation assays have indeed shown that 6.10.2 cells in their nuclear extracts contain a factor that specifically interacts with RRE [34]. These results suggest that the transfected RRE releases repression from the MMTV promoter enabling also low GR concentration to induce MMTV expression.

DISCUSSION

Based on the data of steroid-binding, DNA-binding (both specific and nonspecific), immunoreactivity and electrophoretic mobility, glucocorticoid resistant 6.10.2 cells appeared to contain GR with no obvious physical abnormalities, although the receptor was present in considerably lower concentration as compared to wild-type cells (about 20% as abundant as in wild-type glucocorticoid sensitive 762 cells). Furthermore, the GR mRNA level was proportionally reduced along with the GR protein level in 6.10.2 cells indicating that the lesion causing the reduced GR protein level might be due to a reduction in transcription of the GR gene or stability of the GR mRNA.

The resistance of 6.10.2 cells to glucocorticoid mediated down-regulation of GR mRNA together with the cellular insensitivity to hormonal induction of MMTV mRNA and TAT activity (Figs 3 and 4) demonstrate that both positively and negatively controlled gene expression is nonresponsive in 6.10.2 cells. Miesfeld *et al.* [18] have shown that by expression of cloned GR cDNA in 6.10.2 cells, the mutant phenotype was functionally complemented indicating that the machinery for glucocorticoid responsiveness is still functional in 6.10.2 cells. However, this experiment does not elucidate the functional activity of the endogenous GR present in 6.10.2 cells. In the present study, cellular synthesis of the endogenous GR in 6.10.2 cells was increased 2-fold by treatment with 8-bromo-cAMP. This minor increase in the GR level by 8-bromo-cAMP was sufficient to activate the glucocorticoid responsiveness in 6.10.2 cells as measured by induction of MMTV mRNA and TAT activity as well as the suppression of GR mRNA levels by dexamethasone (Fig. 3). The magnitude of the induction of MMTV mRNA and TAT activity was not as pronounced as in wild-type cells [16]. However, the cAMP-induced GR level was still below the GR level in wild-type cells. In any case, these results demonstrate that the endogenous GR in 6.10.2 cells is indeed biologically active.

An interesting question is why the residual amount of GR present in 6.10.2 cells is not enough to induce a detectable, albeit low, glucocorticoid response. A threshold level of GR required to support the growth inhibition and cytolytic response in mutant lymphoma cells has been established to be in excess of 25% of the wild-type levels in WEH17 cells [35]. Danielsen and Stallcup [36] showed that a novel class of cell variant overcame its inherent sensitivity to glucocorticoids by reversibly down-regulating the level of GR. Other glucocorticoid responses might also require such a threshold level of receptor concentration [12]. Our data suggest that the residual low GR number maintained in 6.10.2 cells is probably below the threshold level required for glucocorticoid induced transcriptional responses. Treatment with 8-bromo-cAMP increased the GR level 2-fold thereby passing a threshold level (Fig. 6).

Cis-acting negative regulatory elements have been reported in association with several steroid

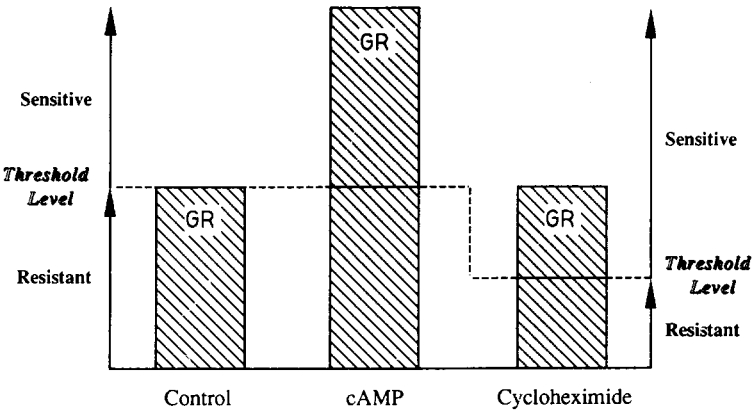

Fig. 6. A hypothetical model for a balance between GR and a repressor protein in regulating glucocorticoid sensitivity.

hormone regulating genes. A *cis*-acting negative element within the ovalbumin promoter region has been shown to be responsible for the cell-specific repression of ovalbumin gene expression, and the effect of steroid hormones is to derepress the promoter [30]. Morley *et al.* [33] have shown that a 96 bp DNA segment of MMTV-LTR 5′ to the GRE sequence represses the inherent activity of the proviral promoter in the absence of glucocorticoids. Moreover, a repressor protein acting on the MMTV promoter and suppressing the GRE enhancer activity has been found in mouse mammary epithelial cells [32]. Removal of such a repressor protein(s) might explain the restoration of glucocorticoid sensitivity in 6.10.2 cells. This could occur following cycloheximide treatment if the repressor or its activity is controlled by a high turnover rate. Lowering of the threshold level controlled by a repressor could explain the restoration of glucocorticoid sensitivity in the 6.10.2 cells (Figs 4 and 6). In fact this seems to be the case, since removal of such a repressor protein by *in vivo* titration with a known negatively acting *cis*-element from the MMTV-LTR, restored glucocorticoid inducibility of MMTV expression (Fig. 5). Thus, it seems likely that the balance between cellular receptor and labile repressor(s) levels determines the sensitivity of target cells to glucocorticoid administration. Changes in concentration or activity of any of these factors in relation to each other may change cellular glucocorticoid sensitivity. Experiments identifying the putative factor(s) in 6.10.2 cells may help us to further characterize the molecular basis of glucocorticoid regulation of gene expression and glucocorticoid resistance.

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