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Decreased Glucocorticoid Receptor Activity Following Glucocorticoid Receptor Antisense RNA Gene Fragment Transfection

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Depression is often characterized by increased cortisol secretion caused by hyperactivity of the hypothalamic-pituitary-adrenal axis and by nonsuppression of cortisol secretion following dexamethasone administration. This hyperactivity of the hypothalamic-pituitary-adrenal axis could result from a reduced glucocorticoid receptor (GR) activity in neurons involved in its control. To investigate the effect of reduced neuronal GR levels, we have blocked cellular GR mRNA processing and/or translation by introduction of a complementary GR antisense RNA strand. Two cell lines were transfected with a reporter plasmid carrying the chloramphenicol acetyltransferase (CAT) gene under control of the mouse mammary tumor virus long terminal repeat (a glucocorticoid-inducible promoter). This gene construction permitted assay of the sensitivity of the cells to glucocorticoid hormones. Cells were also cotransfected with a plasmid containing 1,815 bp of GR cDNA inserted in the reverse orientation downstream from either a neurofilament gene promoter element or the Rous sarcoma virus promoter element. Northern (RNA) blot analysis demonstrated formation of GR antisense RNA strands. Measurement of the sensitivity of CAT activity to exogenous dexamethasone showed that although dexamethasone increased CAT activity by as much as 13-fold in control incubations, expression of GR antisense RNA caused a 2- to 4-fold decrease in the CAT response to dexamethasone. Stable transfectants bearing the GR antisense gene fragment construction demonstrated a 50 to 70% decrease of functional GR levels compared with normal cells, as evidenced by a ligand-binding assay with the type II glucocorticoid receptor-specific ligand [^{3}H]RU 28362. These results validate the use of antisense RNA to GR to decrease cellular response to glucocorticoids.

Patients suffering from severe depression often show an increased activity of the hypothalamic-pituitary-adrenal axis, as indicated by hypersecretion of corticotropin-releasing factor and cortisol, and a premature escape from the cortisol suppressant action of dexamethasone (3, 12). These activities return to normal following successful antidepressant therapy (7). Since neuronal glucocorticoid receptors (GR) are necessary for the negative feedback action of glucocorticoid hormones on the hypothalamic-pituitary-adrenal axis and since antidepressants can modulate the glucocorticoid receptor mRNA content of cells derived from brain areas involved in control of the hypothalamic-pituitary-adrenal axis (17), it is possible that the apparent lack of sensitivity to glucocorticoids seen in patients with depressive illness is due to an abnormality of GR regulation at the level of the limbic-hypothalamic system.

To explore this hypothesis in an animal model, we have attempted to reduce cellular GR levels by introduction of a complementary GR antisense RNA strand, and, before producing transgenic animals, we tested the efficacity of this procedure in cells maintained in culture. Introduction, of either antisense RNA or vectors expressing antisense RNA into eukaryotic cells has been shown to decrease the level of the final product of the complementary-sense mRNA (8, 9, 11, 13, 14). When injected into the cytoplasm of cells, antisense RNA had to complex with the 5′ region of the endogenous mRNA to block its translation (14). However, when expressed in the nucleus, antisense RNAs directed against either the 5′ or the 3′ end of endogenous mRNA were both effective (11, 13). These antisense RNAs presumably form double-stranded RNA, resulting in the inhibition of processing and/or transport of the sense strand RNA from the nucleus to the cytoplasm (11).

To produce antisense RNA, 1,815 bp of the 3′ noncoding region of the GR cDNA was inverted downstream from a human neurofilament gene promoter element or a Rous sarcoma virus (RSV) promoter element. This construction leads to transcription of the antisense strand of the GR cDNA fragment instead of the sense strand. Before creating transgenic mice, we validated this approach to a functional reduction in GR activity by using cell cultures.

The plasmids expressing antisense RNA of a portion of GR were cotransfected with a reporter plasmid carrying the chloramphenicol acetyltransferase (CAT) gene under mouse mammary tumor virus long terminal repeat (MMTV LTR) control. The MMTV LTR confers glucocorticoid inducibility of transcription (13, 14), and the MMTV promoter contains conserved DNA sequences which bind the GR (1, 16, 19). In this system, CAT activity is induced by glucocorticoid (dexamethasone) addition only if GR is present in the cells.

In this work, we examined the effect of the antisense transcripts on GR gene expression in mouse LTK$^-$ cells and mouse neuroblastoma NB41A3 cells. By transfection of the DNA vector directing antisense transcription of the GR gene, the cells themselves produce the anti-mRNA complementary to the GR mRNA. Glucocorticoid-stimulated CAT activity (which is proportional to the GR content) is decreased two- to fivefold when the cells are transfected with the GR antisense RNA. These results show that an antisense

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RNA complementary to the GR can decrease the response of the cells to glucocorticoids.

MATERIALS AND METHODS

Cell cultures. The following cell lines, obtained from the American Type Culture Collection, were used as recipient cells in this study: CV-1 cells (monkey kidney), LTK$^-$ cells (mouse fibroblast), and NB41A3 cells (mouse neuroblastoma). The CV-1 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, the LTK$^-$ cells were grown in minimum essential alpha medium supplemented with 10% fetal calf serum, and the NB41A3 cells were grown in Dulbecco modified Eagle medium-nutrient mixture F-12 (Ham) (1:1) supplemented with 5% fetal calf serum (GIBCO Laboratories). All transfected cultures were maintained at 37°C in a 5% CO_2–95% air atmosphere.

Cell transfection. Recombinant plasmids were expressed either transiently or after stable integration in the host cell genome (5). For transient-expression assays, the recombinant DNA construction (1 to 20 μg) was cotransfected with 2.5 μg of pRSV-LacZ (*Escherichia coli lacZ* gene, which encodes β-galactosidase, fused to the RSV LTR) and with 5 μg of reporter plasmid pMTVCAT (kindly provided by V. Giguère) by the calcium phosphate technique (21). The reporter plasmid consists of the CAT gene under the control of MMTV LTR, which confers glucocorticoid inducibility if GR is present in the cells. At different times after transfection, cells were harvested and assayed for β-galactosidase and CAT activities. Plasmid DNA was precipitated and introduced into cells by incubation for 5 h with 2×10^6 (for LTK$^-$ and NB41A3) or 1×10^6 (for CV-1) cells in 75-cm^2 flasks followed by two washes with 5 ml of HEPES buffer (6.7 mM KCl, 0.14 M NaCl, 9.2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.3]). Dexamethasone (1×10^{-7} M for CV-1 or LTK$^-$ cells; 2×10^{-7} M for NB41A3 cells), if added, was introduced 24 h before harvesting.

Stable transfectants were produced by coprecipitating the recombinant plasmid (20 μg) and a neomycin resistance vector (pRSV NEO; 0.4 μg) with calcium phosphate (19) in 5×10^5 host cells cultured in 75-cm^2 flasks (20). After 24 h, and every 3 days thereafter, the medium was replaced with fresh medium containing the neomycin analog G418 (155 μg/ml for LTK$^-$ cells and 180 μg/ml for NB41A3 cells) to select for neomycin-resistant clones. After 2 weeks, cell colonies were pooled and grown until they were used for ligand binding or for transient-transfection assays with pMTVCAT.

Northern (RNA) blot analysis. RNA was prepared from cells (15×10^6 to 20×10^6) by the guanidium isothiocyanate method (4). Total RNA was separated on 0.8% agarose-formaldehyde denaturing gels and blotted onto nylon filters (Hybond N$^+$; Amersham) prior to hybridization with GR cRNA sense or antisense probes and a β-actin cRNA probe.

GR cRNA sense or antisense probes were produced by T7 or SP6 polymerase runoff transcription, with [^{32}P]UTP, of a 1.8-kb GR cDNA fragment (15) subcloned into plasmid pGEM-1. A β-actin cRNA probe was generated from a 1,500-bp β-actin cDNA *Pst*I fragment inserted into pGEM-1. Filters were prehybridized for 4 h at 42°C (in a mixture of 50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] 6× Denhardt solution, 0.1% sodium dodecyl sulfate [SDS], 50 mM sodium phosphate, 200 μg of yeast tRNA per ml, and 200 μg of denatured salmon sperm DNA per ml) and hybridized at 65°C for 20 h. After hybridization under stringent conditions, the filters were washed twice for 30 min each time in 2× SSC–0.1% SDS at room temperature and twice (1 h each) in 0.1× SSC–0.1% SDS at 70°C. They were wrapped in Saran Wrap and exposed to Kodak X-Omat films with (for filters hybridized with GR cRNA sense or antisense probes) or without (for β-actin cRNA probe) intensifying screens.

CAT assay. Cells were harvested in 40 mM Tris-HCl (pH 7.4)–1 mM EDTA–0.15 M NaCl, pelleted, and resuspended in 0.25 M Tris-HCl (pH 7.8). They were subsequently lysed by three cycles of freezing-thawing and centrifuged, and the supernatants were used for protein, β-galactosidase, and CAT assays. A measure of β-galactosidase activity was obtained by the ability of a supernatant fraction containing 50 μg of protein (determined by the Bradford test [2]) to hydrolyze 0.8 mg of *o*-nitrophenyl-β-D-galactoside in 1 ml of 0.1 mM sodium phosphate (pH 7.0)–10 mM KCl–1 mM $MgSO_4$–50 mM β-mercaptoethanol. The reaction was stopped by the addition of 0.5 ml of 1M Na_2CO_3, and hydrolysis was determined by colorimetry at 420 nm. β-Galactosidase activity was used to normalize the quantity of supernatant subsequently taken for CAT assay. CAT activity was measured in a 150-μl reaction mixture (0.25 M Tris-HCl [pH 7.8], 0.2 μCi of [^{14}C]chloramphenicol [Dupont, NEN], 0.5 mM acetyl coenzyme A) incubated at 37°C for 45 min (6). Acetylated forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography on Whatman LK6D plates. Results are expressed as percent chloramphenicol converted to acetylated forms at constant β-galactosidase activity.

Recombinant plasmids. Plasmids NFL ASGR 2 and RSV ASGR direct the transcription of an antisense RNA complementary to the 3′ noncoding region of the rat glucocorticoid receptor mRNA.

NFL ASGR 2 was constructed by cloning a 1,815-bp fragment of the 3′ noncoding region of the rat GR cDNA in a reversed orientation downstream from the human neurofilament gene promoter. The plasmid (pUC19) containing the human neurofilament L gene promoter (pNF-L, a gift of J. P. Julien [10]) was linearized by digestion with *Hin*dIII, which cleaves at a sequence at the 3′ end of the promoter. Extruding sequences were filled in with the Klenow fragment of DNA polymerase, and a blunted *Xba*I-*Pst*I fragment of the pSG-1 plasmid (a gift of R. Miesfeld; this cDNA clone is a derivative of pRM16, which was described by Miesfeld et al. [15]) was inserted into pNF-L in a reverse orientation. Finally, we added a *Pst*I-*Bam*HI fragment of the VP1 gene of simian virus 40, which contains a polyadenylation signal (Fig. 1).

The second antisense plasmid (RSV ASGR) used in this study consists of the 1,815-bp fragment of the 3′ noncoding region of the rat GR cDNA inserted in the reverse orientation downstream from the RSV LTR promoter. The RSV ASGR plasmid was constructed with the *Ava*I-*Nde*I fragment of NFL ASGR 2 ligated to the *Nde*I-*Hin*dIII fragment of pRSV-Lac (pRSV-Lac encodes β-galactosidase). The neurofilament gene promoter region was removed from NFL ASGR 2 by sequential incubations with *Ava*I, the Klenow fragment of DNA polymerase, and *Nde*I. It was replaced with the RSV LTR promoter removed from pRSV-Lac by sequential digestions with *Hin*dIII, the Klenow fragment of DNA polymerase, and *Nde*I (Fig. 1).

Control plasmids were constructed that were designed to detect any possible inhibitory effect of either the RSV or the NFL promoter sequences which result from competition for available transcription factors in the cell necessary for

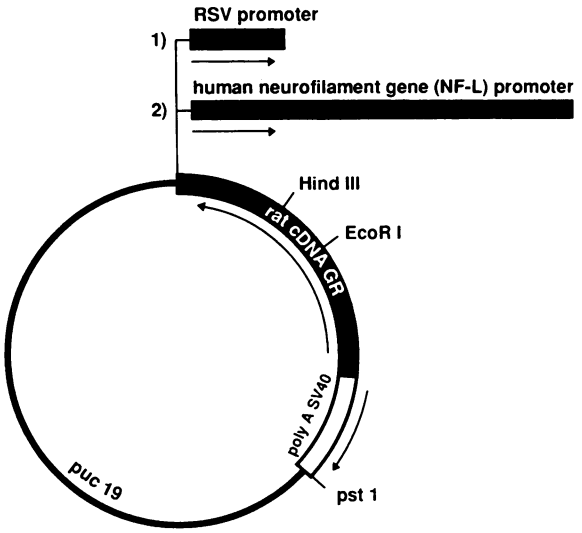

FIG. 1. Schematic representation of the two recombinant plasmids used to direct the transcription of an antisense RNA with a sequence complementary to that of a section of the rat GR mRNA.

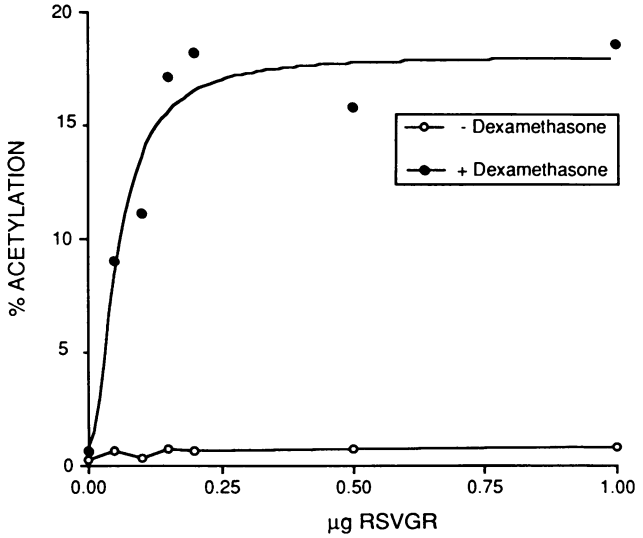

FIG. 2. Dose-response curve in CV-1 cells (which have no detectable GR and are not able to mediate the dexamethasone induction of CAT activity) when the pMTVCAT plasmid is cotransfected with different amounts of a GR expression vector (pRSVGR).

maximal induction of MMTV-CAT. These plasmids were the equivalent of RSV ASGR and of NFL ASGR 2, except that the 1,815-bp GR cDNA was in the correct orientation; they were termed, respectively, RSV SGR and NFL SGR 2.

In vitro binding assays of cytosolic receptor. Glucocorticoid binding was measured with [^{3}H]RU 28362. Cells (1.5×10^6 cells per flask) were rinsed with an isotonic buffer (phosphate-buffered saline [PBS]), scraped in 30 mM Tris–1 mM EDTA–10 mM molybdate–10% (vol/vol) glycerol–1 mM dithiothreitol (TEDGM [pH 7.4]), and sonicated. After centrifugation at 105,000 × *g* for 45 min at 0°C, a sample of the cytosol was incubated with [^{3}H]RU 28362 (specific activity, 77.5 Ci/mmol; concentration range, 0.1 to 10 nM; Dupont, NEN, Boston, Mass.) for 20 to 24 h at 4°C. The amount of nonspecific binding was determined in parallel incubations of the labeled steroid in the presence of a 500-fold excess of unlabeled RU 28362. Sephadex LH20 (Pharmacia) columns (7 by 1 cm, equilibrated with TEDGM buffer), made from 1-ml disposable pipette tips, were used to separate bound from unbound steroid. Following incubation, 100-μl aliquots of the incubated steroids were loaded onto the columns, washed with 100 μl of TEDGM, and eluted with 400 μl of TEDGM into minivials. Each vial was then filled with 5 ml of aqueous counting cocktail (Formula A-963; Dupont, NEN) and counted in an LKB scintillation counter at 40% efficiency.

The values of apparent (molar) dissociation constant (K_d) and the apparent maximum binding capacity (B_{max}) were determined from the Scatchard (18) analyses of the binding data. Protein content was determined by the method of Bradford (2).

RESULTS

Experimental strategy. The strategy used to measure the glucocorticoid sensitivity of the cells was to introduce into the cells a chimeric gene consisting of a glucocorticoid-responsive promoter-enhancer element linked to a reporter gene. We used the MMTV LTR fused to the gene coding for CAT as a reporter plasmid (pMTVCAT) that was able to measure the cellular response to dexamethasone mediated by GR. GR, when activated by glucocorticoid, will bind to a specific DNA sequence localized within the MMTV LTR and induce transcription of the CAT gene. The level of CAT activity is dependent on the cell GR content. This is demonstrated (Fig. 2) by transfection of CV-1 cells (which have no detectable GR and are not able to mediate the dexamethasone induction of CAT activity) with different amounts of a GR expression vector (RSV GR). A dose-response curve shows that MMTV-CAT promoter activity is proportional to the amount of cotransfected GR expression vector. This indicates that a decrease in MMTV-CAT activity could be observed if, in cells which normally contain GR, the translation of endogenous GR mRNA was blocked by an antisense RNA molecule.

Northern blot analysis of GR mRNA sense and antisense strands. Antisense RNA complementary to the 3′ noncoding region of the GR mRNA is produced in LTK$^-$ cells (Fig. 3) and in NB41A3 cells (not shown) stably transfected with RSV ASGR 2, but could not be detected in wild-type cell lines. Endogenous GR mRNA strands were readily detected in both LTK$^-$ and NB41A3 wild-type cells and were reduced by 40 to 80% in cell lines stably transfected with GR antisense gene fragment constructions.

Antisense RNA complementary to the 3′ noncoding region of the GR mRNA decreases the cellular response to dexamethasone. The two antisense plasmids tested here, pNFL ASGR 2 and pRSV ASGR, both direct the transcription of an antisense RNA strand complementary to the 3′ noncoding region of the rat GR mRNA. Although in pNFL ASGR 2 the antisense RNA transcription is under control of a neuron-specific promoter (human neurofilament L), in pRSV ASGR the antisense GR transcription is linked to the RSV LTR promoter. These antisense gene constructions were tested for their ability to decrease the cellular glucocorticoid response both in stable transfectants and in transient-expression assays.

Although both systems gave the same general pattern of results, we observed that stable transfectants are less sensitive to dexamethasone than are transiently transfected

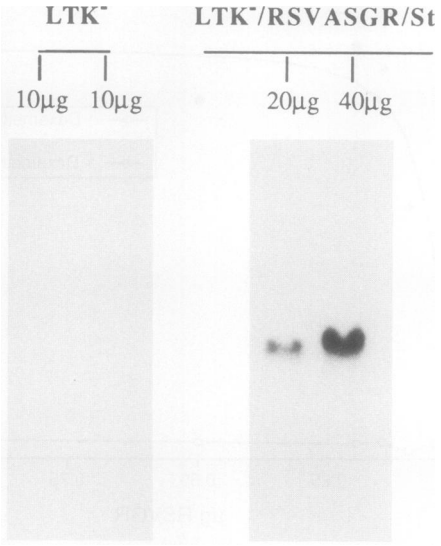

FIG. 3. Northern blot analysis of GR antisense strand formation. Total RNA extracted from 15×10^{10} to 20×10^{10} LTK$^-$ cells or LTK$^-$/RSV ASGR/St cells was analyzed by Northern blot hybridization with a GR cRNA sense probe. No signal was detected in LTK$^-$ cells, and a single band, corresponding to the GR antisense RNA fragment, was detected in LTK$^-$/RSV ASGR/St stable transfectants.

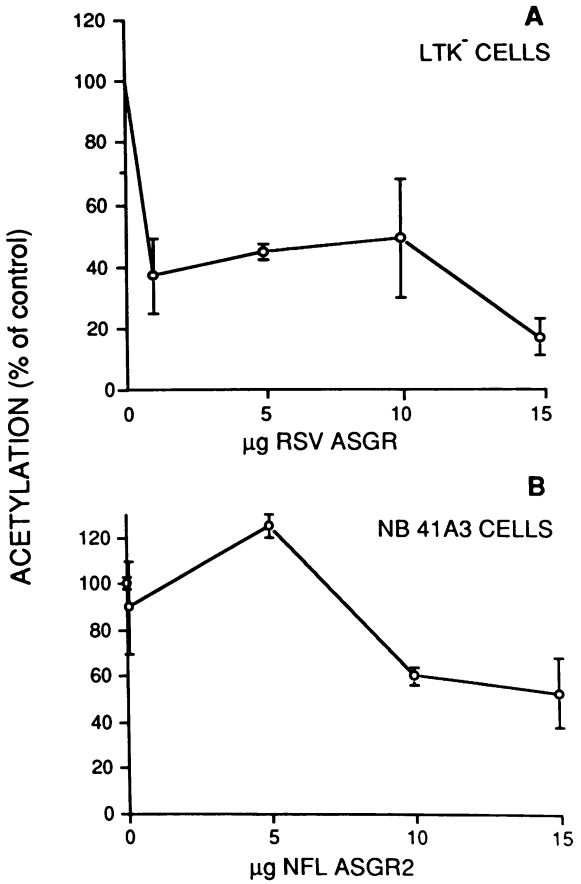

FIG. 4. Promoter activity of the reporter plasmid (pMTVCAT) when transiently cotransfected with RSV ASGR in LTK$^-$ cells (A) or with NFL ASGR 2 in NB41A3 cells (B). The recombinant DNA constructions (1 to 20 μg) were precipitated with pRSV-LacZ and the plasmid reporter pMTVCAT and introduced for 5 h into 2×10^6 LTK$^-$ or NB41A3 cells. Dexamethasone (0.1 μM for LTK$^-$ cells; 0.2 μM for NB41A3 cells) was added 48 h after transfection, and the cells were harvested 24 h later and assayed for CAT activity (at constant β-galactosidase activity for each CAT assay).

LTK$^-$ or NB41A3 cells. In LTK$^-$ cells cotransfected with RSV ASGR (Fig. 4A), the CAT activity is decreased by 50 to 80% compared with that in cells cotransfected with an identical amount of the control vector pUC18. When LTK$^-$ cells that have stably integrated the pNFL ASGR 2 into their genome (labeled LTK$^-$/NFL ASGR 2/St) were transiently transfected with the reporter plasmid pMTVCAT, an 85 to 90% reduction of the CAT response to dexamethasone was seen (Fig. 5). Moreover, stable transfectants of LTK$^-$ cells that have integrated the antisense plasmid RSV ASGR (labeled LTK$^-$/RSV ASGR/St) had a 50% reduced reporter plasmid promoter (MMTV LTR) activity (Fig. 5).

NB41A3 cells, cotransfected transiently with 10 or 15 μg of NFL ASGR 2, are less sensitive to dexamethasone than are cells cotransfected with 10 or 15 μg of the control vector pUC18 (Fig. 4B). In the stable transfectants NB41A3/RSV ASGR/St (NB41A3 cells that have stably integrated in their genome the antisense plasmid pRSV ASGR), transcription from the MMTV LTR promoter of the reporter plasmid (pMTVCAT) was reduced by 70% (Fig. 5).

To control for any reduction in MMTV-CAT expression which could be produced by RSV or NFL promoter elements competing for available transcription factors, either LTK$^-$ or NB41A3 cells (transfected with 5 μg of MMTV-CAT and 2.5 μg of pRSV-LacZ per 2×10^6 cells) were cotransfected with 20 μg of NFL SGR 2 or 20 μg of RSV SGR. No reduction in MMTV-CAT expression was caused by these control plasmids when compared with results for cells not transfected with a control plasmid or transfected with pUC18 as control plasmid (Table 1). These results also demonstrate that addition of nonspecific DNA (pUC18) does not cause any decrease in MMTV-CAT expression when compared with results for cells transfected with MMTV-CAT alone.

The kinetics of MMTV LTR promoter expression in LTK$^-$ and NB41A3 cells when the reporter plasmid pMTV-CAT is cotransfected with 10 μg of pRSV ASGR or 10 μg of pNFL ASGR 2 are illustrated in Fig. 6. In NB41A3 cells, MMTV LTR promoter activity is decreased 48 h after the cotransfection, whereas in LTK$^-$ cells, decreased expression is not seen until 72 h after transfection.

Antisense RNA complementary to the 3′ noncoding region of the GR mRNA decreases glucocorticoid binding in stable transfectant cells. Type II GR binding activity in stable transfectant cells from the NB41A3 cell line was measured to determine, by a functional assay, whether the antisense RNA could decrease the GR concentrations (Fig. 7). Although a maximum of 43.9 fmol of [^{3}H]RU 28362 per mg of protein was bound (K_d = 2.3 ± 0.6 nM) in control cells, glucocorticoid binding was markedly reduced (B_{max} = 25.1 fmol of [^{3}H]RU 28362 per mg protein; K_d = 2.7 ± 0.8 nM) in the stable transfectant cells (NB41A3/RSV ASGR/St). This 50% decrease of the glucocorticoid-binding capacity of these cells compares favorably with the observed reduction of CAT activity in response to dexamethasone.

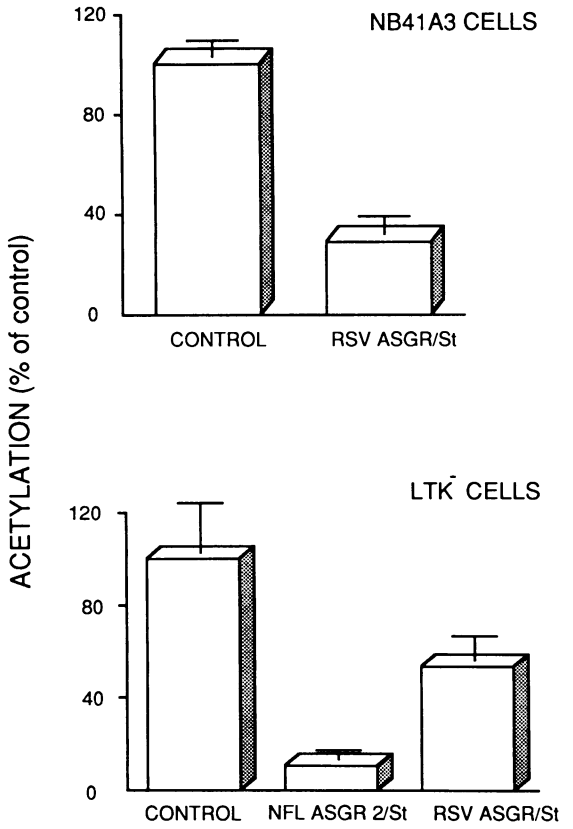

FIG. 5. CAT activity of stable transfectants retransfected with 5 μg of pMTVCAT. The stable transfectants of LTK⁻ cells (labeled LTK⁻/NFL ASGR 2/St or LTK⁻/RSV ASGR/St) and the stable transfectants of NB41A3 cells (labeled NB41A3/RSV ASGR/St) were produced by calcium phosphate coprecipitation of the GR antisense construction plasmid (20 μg) with a neomycin resistance vector (pRSV NEO, 0.4 μg). Clones which had incorporated the recombinant plasmids into the host cell genome were selected by growth in medium containing the neomycin analog G418.

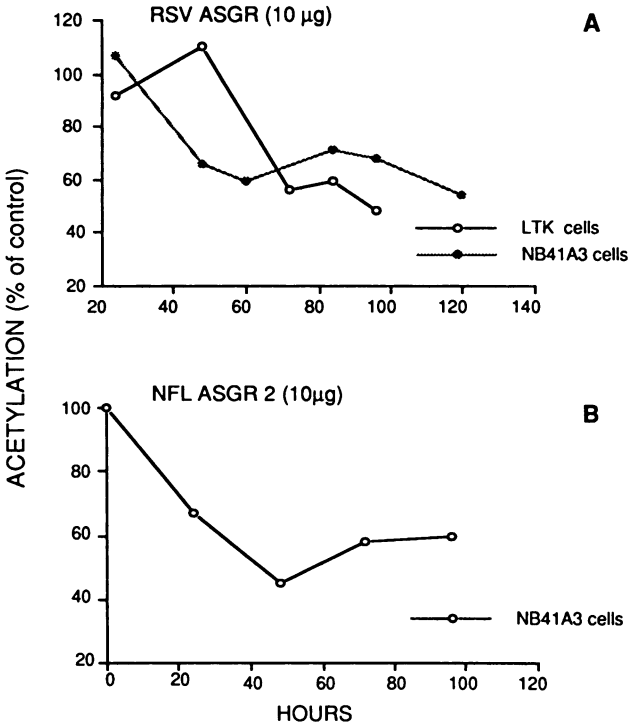

FIG. 6. Kinetics of MMTV LTR promoter expression in LTK⁻ and/or NB41A3 cells when the reporter plasmid pMTVCAT is transiently cotransfected with 10 μg of RSV ASGR (A) or 10 μg of NFL ASGR 2 (B). The GR antisense and reporter plasmids were introduced during a 5-h period into 2 × 10^6 cells (NB41A3 or LTK⁻). Dexamethasone (0.1 μM for LTK⁻ cells and 0.2 μM for NB41A3 cells) was added 24 h before the cells were harvested. At different times, cells were harvested and assayed for CAT activity (at constant β-galactosidase activity for each CAT assay).

DISCUSSION

The experimental strategy used in this study was to measure the response of the cells to dexamethasone that is mediated by the GR with a reporter plasmid, pMTVCAT. The GR, when activated by glucocorticoids, will bind to the MMTV promoter and induce transcription of the CAT gene. We have demonstrated that in the presence of dexamethasone, the resultant CAT activity is proportional to the cellular GR content (Fig. 2). By taking this approach, we can thus evaluate the consequences of any reduction in functional GRs caused by the introduction of a transgene expressing GR antisense RNA. The results presented in this study show that when plasmids designed to produce GR antisense RNA are cotransfected with the reporter plasmid pMTVCAT, a 50 to 80% decrease in functional GRs is obtained, as evidenced by dexamethasone-stimulated CAT activity. These results were obtained in mouse LTK⁻ and NB41A3 cells transfected either transiently or with the transgene stably incorporated into the host cell genome. This decrease of functional GR activity is confirmed by results of a ligand-binding assay with the type II GR-specific ligand ^{3}[H]RU 28362. Both techniques used in this study, i.e., CAT activity and ligand binding, demonstrated a 50 to 70% decrease of functional GR levels in the stable transfectants NB41A3/RSV ASGR/St compared with those in NB41A3 cells. The GR antisense construction transgene is thus expressed when it is stably integrated in the host cell genome, and the GR antisense RNA transcribed effectively diminishes GR production.

The cell lines used in this study are derived from the mouse, whereas the GR antisense RNA we used is complementary to the 3′ noncoding region of rat GR mRNA. Although the nucleotide sequence of the 3′ region of mouse GR cDNA is not yet known, we can presume that there is a high degree of homology between rat and mouse GR cDNA sequences since the rat cRNA probe detects mouse GR

TABLE 1. Effect of control plasmids RSV SGR and NFL SGR 2 on MMTV-CAT expression in LTK⁻ and NB41A3 cells

Control plasmid	CAT activity (% acetylation)[a]	
	LTK⁻ cells	NB41A3 cells
None	100 ± 5	100 ± 6
pUC18	94 ± 5	90 ± 10
RSV SGR	96 ± 6	96 ± 2
NFL SGR 2	92 ± 6	101 ± 9

[a] Results are the mean ± standard error of the mean of three separate experiments.

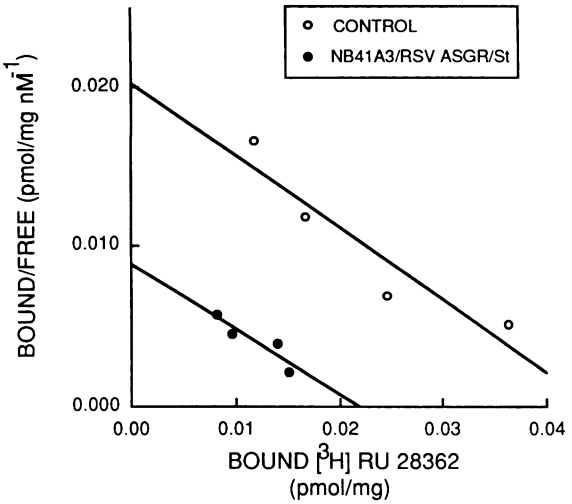

FIG. 7. Scatchard plots of [^{3}H]RU 28362 binding to GR in NB41A3 cells, control cells (stable transfectants without GR antisense plasmid), and NB41A3/RSV ASGR/St cells. The apparent (molar) dissociation constant (K_d) and the apparent maximum binding capacity (B_{max}, expressed as femtomoles per milligram of cytosolic protein) were determined from the Scatchard analyses of the binding data.

mRNA at the same high stringency that we use for rat GR mRNA analysis. The exact mechanism of action whereby the antisense RNA can decrease the GR level in the cells is not known, but different hypotheses may be retained. Transcription of the transiently transfected plasmid or of the stably integrated transgene into RNA must occur in the cell nucleus. Formation of double-stranded RNA, resulting in the inhibition of processing and/or transport of the GR sense RNA from the nucleus to the cytoplasm or in stimulation of GR mRNA degradation, are thus the most probable causes of decreased GR activity, and the decreased GR mRNA concentrations seen in cells stably transfected with the GR antisense transgene support this hypothesis. However, we cannot exclude a cytoplasmic site of action on GR mRNA translation, and the 3′ noncoding region complementarity of the GR antisense RNA could possibly prevent ribosomal dissociation from endogenous mRNA strands.

We observed that the time required to decrease the MMTV LTR promoter activity is 48 to 72 h after the cotransfection of the cells. Functional GRs already present in the cells will not be affected by the antisense RNA, and they are able to activate transcription of the CAT gene under MMTV LTR promoter control. The 48- to 72-h lag period may thus represent the time required for degradation of endogenous GR in the cells before any effect of the GR antisense RNA on new GR formation can be detected.

These results demonstrate that a GR antisense RNA complementary to the 3′ noncoding region of the GR mRNA can decrease the functional GR level in cells in which the GR gene is expressed. It may be possible, by using tissue-specific or inducible promoters, to control the expression of antisense RNA and thus to target either the site or the time at which antisense RNA is supplied in the cell. Although the NFL promoter does not show any tissue specificity in the studies described here, it has been shown to direct gene expression to neurons in vivo (10). It is most likely that immortalized cell lines used in this study are dedifferentiated and have lost their tissue-specific characters. In transgenic animals the site-directed perturbation of the function of a crucial gene such as the GR, which is essential for the normal development and differentiation of cells, could avoid lethal problems, and the NFL ASGR 2 antisense gene construction may be ideal for this purpose.

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