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Transcriptional Transactivation Functions Localized to the Glucocorticoid Receptor N Terminus Are Necessary for Steroid Induction of Lymphocyte Apoptosis

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Genetic studies have suggested that transcriptional regulation of specific target genes (by either induction or repression) is the molecular basis of glucocorticoid-mediated lymphocyte apoptosis. To examine the role of transcriptional regulation more directly, we developed a complementation assay utilizing stable transfection of wild-type (wt) and mutant (nt^i) glucocorticoid receptor (GR) cDNA constructs into a GR-deficient S49 murine cell line (7r). Our data confirm that the level of functional GR is rate limiting for S49 apoptosis and moreover that the GR amino terminus (N terminus), which has been deleted from the nt^i GR, is absolutely required for complementation in this system. Surprisingly, we found that at physiological levels of receptor, expression of the nt^i GR in cells containing wt GR results in enhanced dexamethasone sensitivity rather than a dominant negative phenotype. One interpretation of these data is that DNA binding by wt-nt^i heterodimers may be functionally similar to that of wt-wt homodimers, indicating that GRE occupancy by at least one transactivation domain may be sufficient to induce the hormonal response. To determine whether acidic activating sequences such as those localized to the GR N terminus are important in the induction of lymphocyte apoptosis, we tested the activity of a chimeric receptor in which we replaced the entire GR N terminus with sequences from the herpes simplex virus VP16 protein. Our results demonstrate that 7r cells expressing VP-GR fusions are indeed steroid sensitive, strongly supporting the idea that S49 apoptosis is dependent on transcriptional regulation of specific genes which respond to acidic activating domains, implying that induction, rather than repression, may be the critical initiating event.

Apoptosis, or programmed cell death, has been described as death from within and is utilized by nature as a means of eliminating unwanted cells. In several systems, apoptosis has been shown to require both RNA and protein synthesis (17, 32, 43, 58), suggesting that this form of cell death is an active process. An apoptotic cell progresses through several discrete morphological changes: membrane blebbing, cytoplasmic and nuclear condensation, fragmentation of DNA into oligonucleosome-sized pieces, and margination of chromatin into discrete masses that are aligned on the inner side of the nuclear membrane (66). In the final stages of apoptosis, the cell breaks up into membrane-bound apoptotic bodies. This apoptotic pathway is responsible for the death of many cells during embryonic and postembryonic development and serves a variety of functions in different systems (66).

The key mechanism through which glucocorticoids exert their biological effects involves an interaction with specific high-affinity intracellular receptor proteins which function as ligand-regulated transcription factors (33). It has been shown that the active form of the glucocorticoid receptor (GR) interacts with specific DNA elements (glucocorticoid response elements [GREs]) near glucocorticoid-responsive promoters (46, 57), resulting in a net increased or decreased rate of transcriptional initiation (67). The effects of glucocorticoids are seen in a wide array of cell types, including those of the immune system. During the early stages of differentiation, immature T lymphocytes undergo apoptosis when exposed to physiological levels of glucocorticoids (28). This is also true for a number of neoplastic T-cell lines derived from human (40) and mouse (23, 25) lymphomas. This cellular response is very dramatic in that some mouse lymphoma cells begin to die within hours after addition of glucocorticoids to the culture medium (3).

The fact that steroid-resistant derivatives of such lymphocytes can be easily isolated has been instrumental in the elucidation of the mechanism of steroid action as well as a means of understanding the biology of apoptosis. S49 is a glucocorticoid-sensitive lymphoid cell line which has been extensively characterized and used for the genetic selection of steroid-resistant derivatives (59). Molecular lesions in the receptor are thought to be responsible for the steroid-resistant phenotype in most mutant S49 cell lines. Several classes of mutations have been identified; the most abundant type are those which do not bind hormone (r^-) due to a loss of the receptor or mutations in the ligand-binding domain (8, 20). Two other classes of GR mutants have been described: those which do not bind DNA (nt^-) (2, 8), and a particularly interesting class of mutants, termed nt^i. The nt^i GR was found to bind hormone, translocate to the nucleus, and bind DNA; however, biochemical analysis revealed that the S49 nt^i GR is a truncated form of the receptor (68). This receptor has an apparent molecular mass of 45 to 48 kDa on sodium dodecyl sulfate (SDS)-gels (13), in contrast to the S49 wild-type (wt) receptor, which has an apparent molecular mass of 94 to 97 kDa (41, 52). Recent cloning of nt^i GR cDNA from two independent S49 nt^i mutants revealed that nt^i GR transcripts encode intact steroid and DNA-binding domains but lack 404 N-terminal residues as result of aberrant RNA splicing between exons 1 and 3 (12). Deletion analysis using GR cDNA expression vectors in transient cotransfection assays has demonstrated that the N-terminal

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domain is required for maximal transcriptional regulatory activity (7, 21, 24, 35). The smallest functionally defined transactivation region in the GR N terminus has a net negative charge and has been named enh2 (21) or tau1 (24). Consistent with previous GR deletion mapping studies, we found that the N-terminally truncated nt^i GR is capable of inducing transcription of a mouse mammary tumor virus (MMTV)-chloramphenicol acetyltransferase (CAT) reporter gene to only 10% that of wt GR (12).

Based on the findings that S49 nt^i cells both are dexamethasone (dex) resistant (68) and express a truncated receptor lacking the N-terminal modulatory domain (12), we proposed that transcriptional induction of GR target genes is required for steroid-mediated apoptosis in S49 cells (12, 34). This is in contrast to recent reports which have suggested that transcriptional repression by GR may be necessary for glucocorticoid-dependent killing of the human T-lymphocyte CEM-7 cell line (39) and the P1798 mouse lymphosarcoma (27). In addition to these studies, Gametchu (18) has proposed that there may be a membrane-bound form of the receptor which is critical for S49 cell death, thereby arguing against the role of the nuclear GR protein in transcriptional regulation of apoptosis. In light of these conflicting studies, we designed experiments to directly address the question of GR-dependent transcriptional induction of apoptosis in S49 cells. Our results indicate that GR is rate limiting for this response and moreover that protein sequences known to function as transcriptional activators are necessary for the onset of apoptosis. These findings support our proposal that glucocorticoid-mediated S49 apoptosis is dependent on induction of specific GR target genes.

MATERIALS AND METHODS

Cell lines. The cell lines S49.A2, S49.55r, and S49.7r were obtained from K. Yamamoto and are derivatives of the cell line S49.1T.B4 (59). S49 cells were grown in Dulbecco modified Eagle (DME) medium containing 10% defined and supplemented calf serum (Hyclone) at 37°C in an 8% CO_2 humidity-controlled incubator.

Construction of expression plasmids. The pRX-neo expression plasmid was made by first removing the CAT gene from pMAMCATneo (Clonetech) by using *Sal*I and then adding *Xba*I linkers to the blunted ends to make pMX-neo. The 2.0-kb *Nde*I-*Xba*I fragment, which contains the MMTV long terminal repeat (LTR) from pMX-neo, was removed, and the 0.37-kb *Nde*I-*Pvu*I fragment (Rous sarcoma virus LTR) from pRSVGR (35) and the 0.15-kb *Pvu*I-*Xba*I fragment (multiple cloning site) from p6R (21) were ligated together to give pRX-neo. pKS-hyg was made by removing the 1.9-kb *Nru*I-*Bam*HI fragment containing the hygromycin resistance gene from a derivative of pLG89 (22) and cloning it into pKS+ (Stratagene). The 2.0-kb *Hin*dIII fragment from pKS-hyg (containing the hygromycin resistance gene) was modified by using *Bam*HI linkers and then ligated into pRX-neo at the *Bam*HI sites to make pRX-hyg. The 2.8-kb *Xba*I fragment of p6RmGR (12) containing the wt mouse GR cDNA was cloned into the *Xba*I site of pRX-neo to form pRmGR-neo. Similarly, the 1.5-kb *Xba*I fragment of p6Rnti (12) containing the nt^i GR cDNA was cloned into the *Xba*I site of pRX-neo to form pRnt^i-neo. pRnt^i-hyg was constructed by cloning the 1.5-kb nt^i GR cDNA-containing fragment from pRnt^i-neo into the *Kpn*I-*Xho*I sites of pRX-hyg. The pRN$^+$GR-neo expression plasmid contains two new restriction sites introduced by site-specific mutagenesis using the method of Kunkel (29) which flank the GR N-terminal domain, *Bgl*II at nucleotide position 121 and *Nco*I at nucleotide position 1228. pRN$^-$GR-neo was constructed by removing the 1.1-kb *Bgl*II-*Nco*I fragment from pRN$^+$GR-neo and replacing it with a 21-bp *Bgl*II-*Nco*I adaptor.

Whole-cell hormone binding assay. GR levels were assayed in whole cells as described previously (41), with slight modifications. Briefly, logarithmically growing cells were harvested and resuspended in duplicate reaction mixtures containing 10^7 cells in 0.5 ml of PD buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). [^{3}H]dex (94 Ci/mmol; Amersham), was added to a final concentration of 2×10^{-8} M. Nonspecific binding was assayed by adding 2×10^{-5} M unlabeled dex to the suspensions. Cells were incubated at 37°C for 1 h and then washed three times by resuspension and centrifugation in PD buffer. Radioactivity in whole-cell pellets was assayed by liquid scintillation counting. The counts per minute obtained were normalized to total cellular protein content measured by the BCA protein assay (Pierce). The dex-binding values are an average of duplicate samples derived from three separate experiments.

Stable transfections and soft agar cloning. Logarithmically growing cells were harvested and washed twice in HBS buffer (5 mM KCl, 137 mM NaCl, 6 mM glucose, 21 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES, pH 7.05], 0.7 mM Na_2HPO_4) and then resuspended in HBS buffer at a final concentration of 5×10^6 cells per ml. To each cuvette, 100 μg of GR expression plasmid (50 μg of supercoiled, 50 μg of linear) was added to 1 ml of the cell suspension. Electroporation was carried out at 225 or 275 mV and 1,180 or 800 μF, respectively, using a Cell Porator (Bethesda Research Laboratories). Cells from each cuvette were allowed to recover in 20 ml of conditioned medium for 24 h, after which G418 (Geneticin; GIBCO) was added at 1 mg/ml (active) or hygromycin B (Calbiochem) was added at 1.5 mg/ml (final concentration). After 48 h of selection in liquid culture, the cells were then plated in soft agar (50). This was done by making a fresh stock of filter-sterilized plating medium containing 50% preconditioned growth medium, 30% fresh DME, 10% fresh defined calf bovine serum, G418 or hygromycin B (at the concentration indicated above), and antibiotics to which autoclaved FMC SeaPlaque agarose (6% stock) was added to a final concentration of 0.5%; this was kept at 37°C until ready for use. A bottom layer of plating medium was poured (24 ml/150-cm^2 tissue culture dish) and allowed to solidify at 4°C for 11 min and then for 10 min at room temperature. Cells were centrifuged at 1,500 rpm for 10 min, resuspended in 12 ml of plating medium, and then carefully layered over the bottom agar. This top agar was allowed to solidify, and the plates were then incubated at 37°C. Drug-resistant colonies were visible 7 to 10 days later and picked into six-well dishes with 3 ml of conditioned medium containing G418 or hygromycin B.

Transient transfection assays. Transfections into S49 cells were performed by electroporation as described above, using 50 μg of the reporter plasmid pMM-CAT, which includes 1.4 kb of the MMTV LTR linked to the CAT gene (54), or a heterologous promoter construct, GTCO, containing a simple GRE linked to the thymidine kinase promoter (10). Plasmid pEQ176, a derivative of pON1 (62), containing the bacterial β-galactosidase gene transcribed from the cytomegalovirus enhancer/promoter was included as an internal control (10 μg). Following electroporation, cells from a single cuvette were split into two equal portions and plated in DME containing 10% defined calf bovine serum (or 5% fetal bovine serum with CV-1 cells) with or without 10^{-6} M

dex. After 24 to 48 h in culture, the cells were harvested and protein extracts were prepared and normalized to the same protein concentration. One portion of the extract was used for β-galactosidase assays (53), and another was used for CAT assays as described elsewhere (48). The CAT reaction mixture was incubated at 37°C for 16 h, and acetylated forms of chloramphenicol were separated by thin-layer chromatography on silica plates in 95:5 chloroform-methanol. Percent conversion of [^{14}C]chloramphenicol into mono- and diacetylated forms was determined by Betascope (Betagen) scanning of the thin-layer chromatography plate.

Receptor affinity labeling. Covalent labeling of GRs with [^{3}H]dexamethasone 21-mesylate (dex-mesylate; 44 Ci/mmol; NEN) was performed as described by Rehmus et al. (52). Cells were washed twice with PD buffer and then resuspended in PD buffer at a concentration of 5×10^7 cells per 0.45 ml. Duplicate cell suspensions were incubated with 50 nM [^{3}H]dex-mesylate in the presence or absence of 100-fold-excess unlabeled dex for 4 to 6 h at 4°C. The cells were then pelleted, and the supernatant was discarded. Cytosolic extracts were prepared by freezing the cell pellets at −80°C for 15 min and thawing each cell pellet in 20 μl of hypotonic buffer (25 mM Tris [pH 8.2], 1 mM EDTA, 10% glycerol, 20 mM Na_2MoO_4) at 4°C. The supernatant was recovered by centrifugation in an Eppendorf microfuge at 4°C for 15 min. The protein content of each cytosolic extract was determined by the BCA protein assay (Pierce), and equal amounts of protein were run on a 10% polyacrylamide gel under reducing and denaturing conditions (SDS-polyacrylamide gel electrophoresis [PAGE]) (30). Gels were fixed in a solution of glacial acetic acid-methanol-H_2O at a ratio of 1:3:6 for 1 to 3 h and then impregnated with En^{3}Hance (NEN) according to the manufacturer's protocol. Gels were dried and then exposed to Kodak XAR-5 film at −80°C for 7 to 14 days.

RESULTS

Expression of wt GR in a receptor-deficient S49 cell line restores dex sensitivity. To directly determine what role the GR N terminus has in regulating S49 apoptosis, we developed a genetic complementation assay by stably transfecting GR cDNA into an S49 cell line which contains negligible levels of endogenous GR and is resistant to the effects of dex. S49.7r cells (referred to as 7r) were originally isolated from the steroid-sensitive wt cell line S49.1T.B4.1A (here designated A2) by Sibley and Tomkins (59). 7r cells contain approximately 30% the amount of immunologically cross-reactive receptor (41) yet less than 2% the level of hormone binding activity of the parental A2 cell line (Table 1). Consistent with these observations, Miesfeld et al. (36) have shown by Northern (RNA) analysis that 7r cells express a low level of GR mRNA. It therefore appears that 7r cells express a mutant GR (r^-) from one allele (36, 37, 41) and that the other allele is not transcribed.

If the dex-resistant phenotype of 7r cells is the result of GR mutations alone, then it should be possible to complement this defect by stable expression of wt GR cDNA. This was done by transfecting the pRmGR-neo expression plasmid into logarithmically growing 7r cells and selecting for growth in G418. Isolated clonal cell lines were further expanded and then screened for the presence of GR, using hormone binding as a functional assay. A summary of these results is shown in Table 1. To further document that GR expression in the transfected 7r cells (7.G cell lines) was derived from integrated plasmid, Southern and Northern analyses were performed. These results established that most 7.G cell lines contain only a single copy of integrated plasmid DNA and that the GR-positive cell lines express full-length GR cDNA transcripts (data not shown).

TABLE 1. Relative dex-binding values of transfected S49 cell lines[a]

Cell line	GR genotype		Dex binding
	Parental	cDNA	
A2	wt	None	1.00
A.G11	wt	wt	1.61
7r	r^-	None	0.02
7.neo2	r^-	None	0.02
7.hyg4	r^-	None	0.02
7.G9	r^-	wt	1.85
7.G6	r^-	wt	3.78
55r	nt^i	None	1.59
55.n1	nt^i	nt^i	3.91
7.n8	r^-	nt^i	0.63
7.n8.n7	7.n8	nt^i	3.81
A.n14	wt	nt^i	3.61
7.G9.n14	7.G9	nt^i	3.00
7.GVP13	r^-	VP16 fusion	1.04
7.GVP16	r^-	VP16 fusion	1.06

[a] The genotype refers to the endogenous GR gene (parental) or the transfected GR sequences (cDNA). To control for any effects that the neomycin- or hygromycin-resistant gene products may have on the dex-sensitive phenotype (9), 7r cells were transfected with the vector backbone alone (pRX-neo and pRX-hyg). Representative neomycin- and hygromycin-resistant cell lines (7.neo2 and 7.hyg4) were found to be completely dex resistant and indistinguishable from 7r cells (data not shown).

Receptor protein analysis in the 7.G cell lines was carried out by affinity labeling with [^{3}H]dex-mesylate, a high-affinity covalent ligand of GRs (60, 61). Since this labeling method is specific for receptor molecules capable of binding steroid, the small amount of defective GR present in the 7r cells is not detected in this assay. However, dex-mesylate covalently binds to several non-GR proteins present in 7r cells; therefore, whole-cell extracts were labeled in either the presence or absence of excess unlabeled dex to identify GR-specific bands. Figure 1A shows results from an autoradiogram in which GR protein was labeled in wt A2 and dex-resistant 7r cells. It can be seen by this qualitative assay that A2 cells, but not 7r cells, contain a 97-kDa protein specifically labeled by dex-mesylate (arrow). Figure 1A also shows that two 7.G derivatives, 7.G6 and 7.G9, contain high and low amounts of GR protein, respectively. Identification of these bands as GR was confirmed by immunoprecipitations using two different GR-specific antibodies (data not shown). The dex sensitivity of the 7.G stable cell lines compared with those of A2 and 7r cells was determined by growing the cells in the presence of 10^{-6} M dex over a 3-day period. As can be seen in Fig. 1B, wt A2 cells are dex sensitive and 7r cells are completely dex resistant. In contrast to the parental 7r cells, 7.G6 and 7.G9 cell lines are dex sensitive, but to different degrees. 7.G6 cells, which contain high levels of GR (Table 1 and Fig. 1A), are more sensitive to dex than are wt A2 cells, while 7.G9 cells have a dex sensitivity similar to that of A2 cells. These data demonstrate that expression of wt GR cDNA in 7r cells is sufficient to complement the dex-resistant phenotype and moreover that dex sensitivity is correlated to the level of GR expression.

Overexpression of nt^i GR does not complement the dex-resistant phenotype. Since the nt^i GR is partially active (12) and GR is rate limiting within physiological limits (51, 64),

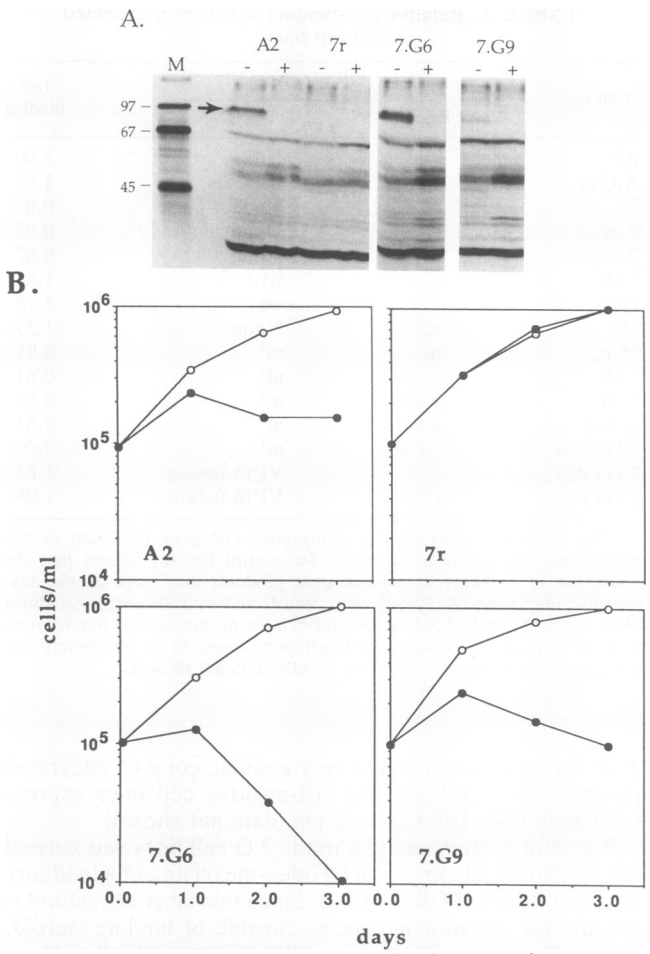

FIG. 1. Wild-type GR protein expression in 7r transfectants. (A) Autoradiograph of an SDS-polyacrylamide gel containing cytosolic extracts from S49 mouse lymphoma cells labeled with [^{3}H]dex-mesylate in the absence (−) or presence (+) of 100-fold-excess unlabeled dex. The 97-kDa affinity-labeled wt GR receptor is indicated by an arrow. Molecular weight standards (M) are ^{14}C-labeled phosphorylase *b* (97.4 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa). (B) Growth curves of cells seeded at 10^5 cells per ml in the absence (open circle) or presence (closed circle) of 10^{-6} M dex. The number of viable cells was determined over a 3-day period on the basis of trypan blue exclusion (45).

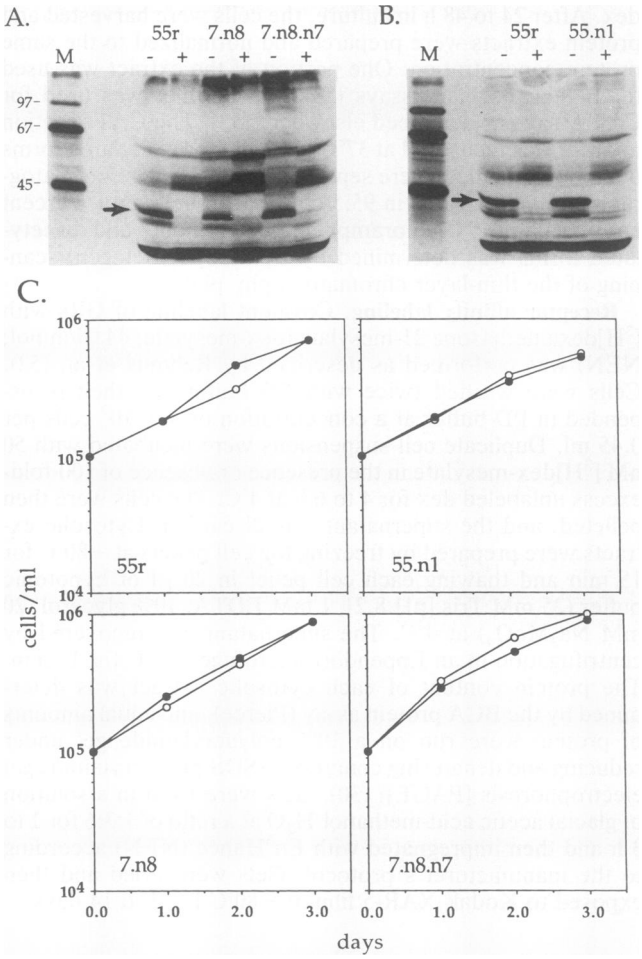

FIG. 2. Overexpression of nt^i GR in S49 cells. Autoradiograph showing [^{3}H]dex-mesylate-labeled receptors from the nt^i cell line 55r and two 7.nt^i GR transfectants, 7.n8 and 7.n8.n7 (A), and 55r and 55.n1 cells, the latter from a 55r transfectant which expresses nt^i GR cDNA (B). Labeling and SDS-PAGE were as described for Fig. 1A. The arrow indicates the affinity-labeled 45-kDa truncated nt^i receptor. (C) Growth curve analyses of the same cell lines as in panel A grown in the absence (open circles) or presence (closed circles) of 10^{-6} M dex.

we tested whether it was possible to increase target gene responsiveness in nt^i GR-containing cells by elevating nt^i GR expression. This was done either by integrating multiple copies of nt^i GR cDNA into 7r cells (using two different expression vectors, pRnt^i-neo and pRnt^i-hyg) or by stably transfecting the nt^i cell line 55r, with nt^i GR cDNA.

We first characterized 7r cell lines expressing nt^i GR cDNA (7.n8 and 7.n8.n7). Protein analysis (Fig. 2A) revealed that the size of the receptor in the 7.n8 and 7.n8.n7 cell lines is approximately 45 kDa and identical to the size of the nt^i GR in 55r cells (12). Growth curve analysis (Fig. 2C) demonstrated that nt^i GR expression in 7.n8 and 7.n8.n7 cells did not result in a dex-sensitive phenotype, even though the level of dex binding in 7.n8.n7 is similar to that found in 7.G6 (Table 1). Similarly, the S49 nt^i cell lines, 55r and 55.n1, are completely dex resistant (Fig. 2C) despite the relatively high level of nt^i GR protein being expressed (Table 1 and Fig. 2B).

To measure the level of transcriptionally active GR in 7r stable cell lines expressing wt or nt^i GR, we performed transient transfection assays using either the pMM-CAT reporter plasmid or a heterologous promoter construct, GTCO (10), containing a simple GRE linked to the thymidine kinase promoter. The results shown in Fig. 3 demonstrate that while the parental 7r cells do not express a transcriptionally active GR, 7.G6 cells express sufficient GR to induce CAT activity 68- and 23-fold with use of pMM-CAT and GTCO, respectively. In contrast, we found that the induction of either pMM-CAT or GTCO by the nt^i GR present in 7.n8.n7 cells was only ~10% that of wt GR, which is consistent with the observed transcriptional regulatory activity of this mutant GR by transient cotransfection assays in CV-1 cells (12).

Coexpression of wt and nt^i GR results in enhanced dex sensitivity. Several studies have shown that GRs bind as dimers to DNA (47, 63). In addition, chemical cross-linking

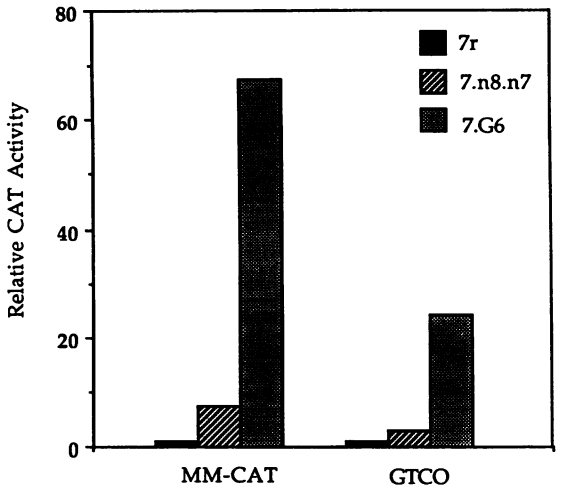

FIG. 3. Functional analysis of wt and nt^i GR expressed in 7r stable lines. Transient transfections using the reporter plasmid pMM-CAT or GTCO were performed as described in Materials and Methods. The value for CAT activity is corrected for β-galactosidase activity and is expressed as a fold induction relative to the parental 7r cells, which have no dex-inducible CAT activity in this assay. These results represent the average of at least three separate experiments.

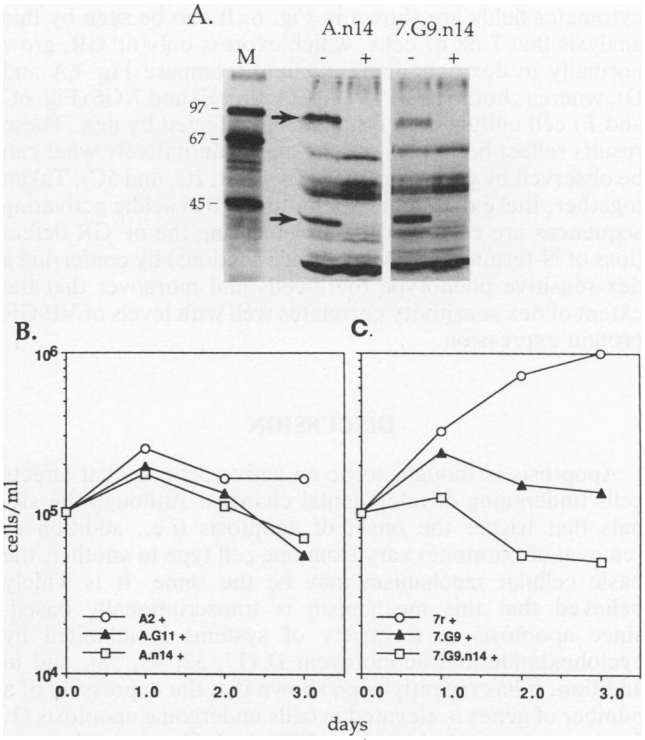

FIG. 4. Coexpression of wt and nt^i GR in S49 cells. (A) Autoradiographs of an SDS-polyacrylamide gel containing labeled extracts prepared from wt A2 cells transfected with nt^i GR cDNA (A.n14) or 7r cells expressing both wt and nt^i GR cDNA (7.G9.n14). The cells were labeled with [^{3}H]dex-mesylate in the absence (−) or presence (+) of 100-fold-excess unlabeled dex as described for Fig. 1A. The arrows show both the wt receptor (97 kDa) and the truncated nt^i receptor (45 kDa). (B and C) Growth curve analyses of A2 cells (wt/−) and two A2 transfectants. (B) A.G11 (wt/wt) and A.n14 (wt/nt^i); (C) 7r (r$^-$) and two 7r transfectants, 7G.9 (wt) and 7.G9.n14 (wt/nt^i). All of the cells were grown in 10^{-6} M dex (+) over a 3-day period and counted as described for Fig. 1B.

experiments have demonstrated that GR homodimers can form in the absence of DNA (65) and that a potential dimerization domain maps to the carboxy-terminal region (15). Therefore, transfected cells which express both nt^i and wt GR may be dex resistant as a result of a dominant negative effect of the nt^i GR. This could be due to nonfunctional nt^i GR homodimers bound at target gene GREs or to the formation of nt^i-wt GR heterodimers which may have reduced transcriptional regulatory activity. For these experiments, 7.G9 cells were transfected with the pRnt^i-hyg expression plasmid, and wt A2 cells were transfected with the plasmid pRnt^i-neo. Representative cell lines (7.G9.n14 and A.n14) were chosen for further analysis. As can be seen in Table 1, 7.G9.n14 and A.n14 contain higher levels of dex binding than do the parental cell lines. An audioradiogram of an SDS-polyacrylamide gel using extracts labeled with dex-mesylate (Fig. 4A) confirmed that A.n14 and 7.G9.n14 express both the wt and nt^i receptor proteins and that the ratios of nt^i to wt GR are approximately 1:1 and 3:1, respectively, as judged from band intensities.

Surprisingly, growth curve analyses (Fig. 4B and C) revealed that expression of the nt^i GR in cells containing wt GR was not deleterious to GR function and did not result in a dominant negative phenotype. In fact, it appears that cells coexpressing nt^i and wt GR are more dex sensitive than cells expressing only wt GR (compare A2 with A.n14 and 7.G9 with 7.G9.n14). At this level of nt^i GR expression, the observed enhancement of dex sensitivity in A2 cells is similar to that seen in cell lines containing additional wt GR (compare A.G11 with A.n14). Thus, these data demonstrate that at physiological levels of receptor, nt^i GR expression does not result in a dominant negative phenotype but rather, nt^i GR augments dex sensitivity when expressed in cells containing wt GR.

Expression of a VP16-GR fusion receptor restores dex sensitivity to 7r cells. Since the transcriptional regulatory functions of the GR N terminus have not been well characterized, we constructed a chimeric receptor in which the 370 GR N-terminal residues were replaced by the carboxy-terminal acidic activation domain of the herpes simplex virus VP16 protein (Fig. 5A). This portion of VP16 has been extensively studied and shown to function as a strong transcriptional activator when fused to a heterologous DNA-binding domain (55). Using transient cotransfection assays into CV-1 cells with the pMM-CAT reporter plasmid, we found that the VP-GR chimeric receptor functions as well as or better than full-length wt GR (Fig. 5B).

7r cells stably expressing the VP-GR fusion protein were isolated (7.GVP13 and 7.GVP16) and characterized by dex-binding assays (Table 1). The growth curves shown in Fig. 5C demonstrate that both 7.GVP13 and 7.GVP16 are sensitive to growth in dex-containing media. The level of dex sensitivity of 7.GVP13 and 7.GVP16 is consistent with the low amount of functional receptor in these two cell lines (Table 1). Moreover, the growth curves of 7.GVP16, 7GVP13, and 7.G9 (Fig. 1B) and the level of functional GR as analyzed by transient transfection assays using pMM-CAT (data not shown) are very similar.

To examine apoptosis in these cells more directly, the relative dex sensitivities of 7.n8.n7, 7.VP13, and 7.G6 were compared by analyzing the number of viable cells (trypan blue negative) after 72 h of growth in the presence or absence of 10^{-6} M dex. Photomicrographs of representative hema-

cytometer fields are shown in Fig. 6. It can be seen by this analysis that 7.n8.n7 cells, which express only nt^i GR, grow normally in dex-containing medium (compare Fig. 6A and D), whereas both 7.GVP13 (Fig. 6B and E) and 7.G6 (Fig. 6C and F) cell cultures are dramatically affected by dex. These results reflect both qualitatively and quantitatively what can be observed by growth analysis (Fig. 1B, 2C, and 5C). Taken together, these data indicate that the VP16 acidic activating sequences are capable of complementing the nt^i GR defect (loss of N-terminal transactivating functions) by conferring a dex-sensitive phenotype to 7r cells and moreover that the extent of dex sensitivity correlates well with levels of VP-GR protein expression.

DISCUSSION

Apoptosis is thought to be an active process that affects cells undergoing developmental changes. Although the signals that trigger the onset of apoptosis (i.e., addition or removal of hormone) vary from one cell type to another, the basic cellular mechanism may be the same. It is widely believed that this mechanism is transcriptionally based, since apoptosis in a variety of systems is inhibited by cycloheximide and actinomycin D (17, 32, 43, 58), and in addition, it has recently been shown that the expression of a number of genes is elevated in cells undergoing apoptosis (1, 5). In support of this idea, Ellis and Horvitz (14) have genetically identified a series of genes in *Caenorhabditis elegans* whose products are required for cells to undergo developmentally programmed apoptosis. These data have led to the proposal that altered transcription of specific genes, by either repression or induction, is the key regulatory event in apoptosis.

Steroid-induced apoptosis in S49 cells provides a unique system in which to study this response, since much is already known about the mechanism of steroid hormone action (34, 67). We have used cloned GR cDNA sequences, coupled with recent improvements in DNA transfection by electroporation, to directly test the proposal made by Yamamoto et al. (68) 15 years ago that GR is the key mediator of dex-induced S49 cell death. Our data demonstrate that a functional copy of wt GR is necessary and sufficient to complement the dex-resistant phenotype of 7r (r$^-$) cells. In addition, we show that the level of wt GR is rate limiting, since cells which express high levels of GR (7.G6) are more dex sensitive than cells expressing low levels of GR (7.G9).

One of the important questions regarding steroid-mediated S49 apoptosis concerns the mechanism of GR-regulated cell death, specifically with regard to the requirement of transcriptional induction versus transcriptional repression. The data presented here support the role of GR-mediated transcriptional induction in apoptosis in two ways. First, we show that the nt^i GR is incapable of initiating the apoptotic pathway in 7r cells expressing high levels of nt^i GR (55r, 55.n1, 7.n8, and 7.n8.n7). It has been shown that an nt^i-like N-terminally truncated GR functions normally in repressing transcription from both the bovine prolactin (38) and the α-subunit chorionic gonadotropic (44) promoters, suggesting that the GR N terminus is not required for transcriptional repression of at least these two genes. On the basis of these results, nt^i GR-containing cells should be dex sensitive if transcriptional repression were occurring at a simple negative GRE (56). Second, our results using the VP-GR fusion receptor demonstrate that replacement of the GR N terminus with the herpes simplex virus VP16 transactivation domain

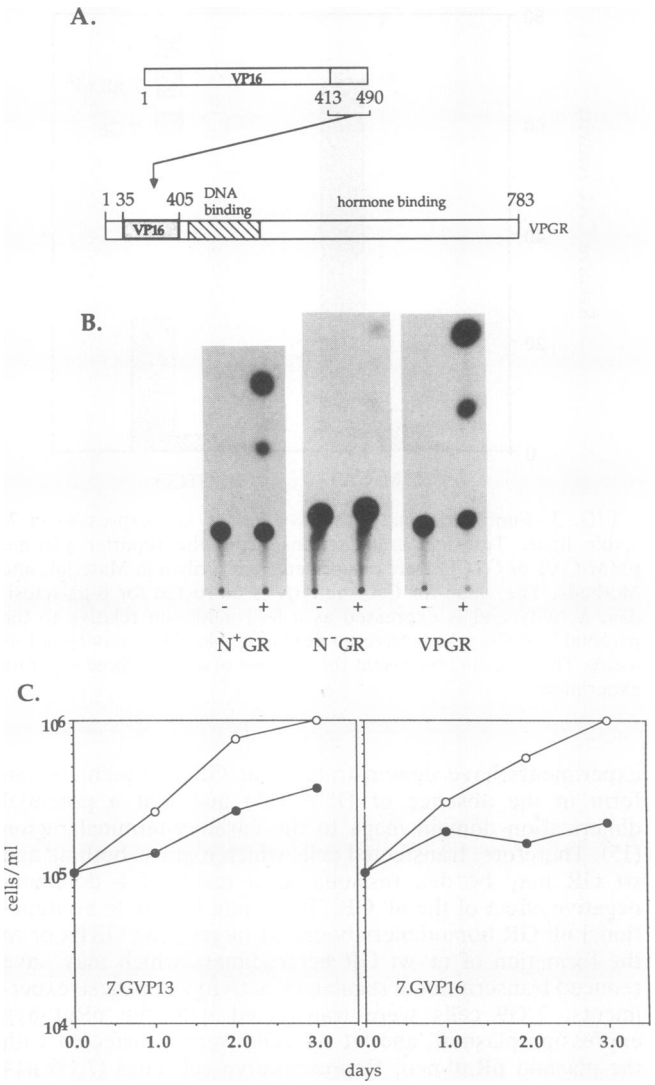

FIG. 5. Expression of a VP-GR fusion protein in 7r cells. (A) Construction of the VP-GR expression plasmid. pRVPGR-neo was made by cloning 233 nucleotides of VP16 cDNA (nucleotides 8 to 241 of pCRF-1 [55]) into the *Bgl*II-*Nco*I sites of the pRN$^+$GR-neo expression plasmid. Expression from this plasmid results in the production of a VP-GR fusion protein in which the N terminus of GR is replaced by the carboxy-terminal 77 amino acids of VP16. (B) The VP-GR fusion protein functions as a steroid-regulated transcriptional activator. CAT assay results are from CV-1 cotransfections using the expression plasmids pRN$^+$GR-neo (containing the entire GR N terminus), pRN$^-$GR-neo (deleted for GR N-terminal residues 36 to 404), and pRVPGR-neo and a pMM-CAT reporter plasmid containing 1.4 kb of the MMTV LTR linked to the CAT gene. Transfected CV-1 cells were grown in the absence (−) or presence (+) of 10^{-6} M dex for 48 h. (C) Dex sensitivity of 7r cells expressing the VP-GR fusion protein. Shown are growth curve analyses of two 7r transfectants, 7.GVP13 and 7.GVP16, grown in the absence (open circles) or presence (closed circles) of 10^{-6} M dex over a 3-day period.

is sufficient to confer a dex-sensitive phenotype (7.GVP13 and 7.GVP16). This result strongly argues for the importance of transcriptional induction in apoptosis, since the mechanism of VP16 transactivation is thought to be the recruitment of limiting factors such as TFIID (26) and TFIIB (31) to an active promoter complex, resulting in increased rates of

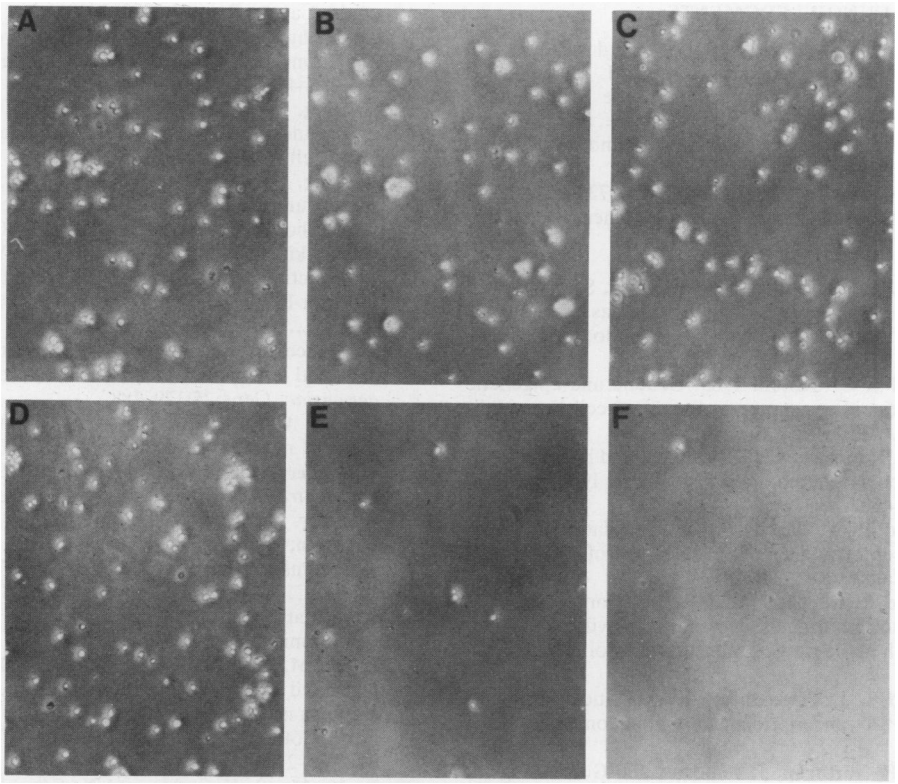

FIG. 6. Photomicrographs showing the dex sensitivities of 7r stable transfectants. The three cell lines 7.n8.n7 (A and D), 7.GVP13 (B and E), and 7.G6 (C and F) were seeded at 1.5×10^5 cells per ml and grown in the absence (A to C) or presence (D to F) of 10^{-6} M dex for 72 h. Cells were stained with trypan blue and loaded onto a hemacytometer before being photographed. Each panel shows the number of live (and dead) cells in 2×10^{-5} ml of cell culture as determined by the area of the hemacytometer field.

transcription. In addition, a type of repression called squelching (49) is probably not involved in the apoptotic response because specific DNA binding has been shown to be absolutely required for S49 cell death (8), and in most cases squelching would act independently of DNA binding (49). However, it is still possible that transcriptional repression could be occurring at composite GREs in S49 cells if GR and VP-GR were interacting with other transcription factors such as AP1, similar to what was found in the analysis of proliferin gene repression by dex (11).

Results from a recent study suggested that transcriptional induction may be dependent more on the number of proteins bound to the promoter than on the number of acidic activation domains present in the complex (42). We and others have observed that the intracellular level of GR influences the magnitude of the steroid response (Fig. 1) (4, 19, 64), suggesting that under normal physiological conditions, GR is a rate-limiting factor. However, we also found that coexpression of wt and nt^i GR in the same cell results in an increase in dex sensitivity rather than in a dominant negative phenotype (A.n14 and 7.G9.n14 in Fig. 4B and C). Since the level of nt^i GR being expressed is comparable to the level of wt GR in A.n14 and 7.G9.n14 cells (Fig. 4A), it could be that these cells contain nt^i-wt GR heterodimers. For example, comparison of the dex sensitivity of A2 (wt/−) cells with those of A.G11 (wt/wt) and A.n14 (wt/nt^i) cells indicates that the additional amount of GR (wt or nt^i) increases dex sensitivity (Fig. 4B and C), suggesting that if wt-nt^i GR heterodimers are forming, they are functional. This could be analogous to the results described by Oliviero and Struhl (42), who show that expression of Jun in cells containing Fos-GCN4 fusion proteins results in the same level of transcriptional induction as when Jun-GCN4 fusions are coexpressed with Fos-GCN4 proteins. In this case, Jun would be like nt^i GR and Fos-GCN4 (or Jun-GCN4) would be similar to wt GR.

Recently, Nazareth et al. (39) reported results of transient transfections which indicate that glucocorticoid-regulated cell death of human CEM cells requires only GR sequences in the first finger of the DNA-binding domain and that the N terminus appears dispensable for CEM apoptosis. They proposed that CEM cell death is the result of transcriptional repression of growth-stimulating genes. Our results using stable transfections of murine S49 cells were quite different in that the elevated expression of nt^i GR in 7.n8.n7 cells was shown to be sufficient for low-level induction of GRE-containing reporter plasmids but not for the control of dex-induced S49 cell death. Since the GR N terminus appears not to be required for transcriptional repression of some genes (38, 44), and the VP16 sequences that we used are thought to function as transcriptional activators (6), we propose that dex-induced S49 cell death is at least partially dependent on transcriptional induction. One explanation for the differences between our results for S49 cells and those of Nazareth et al. (39) for CEM cells could be that distinct mechanisms of apoptosis may exist in these two cell lines, as has been suggested (16). More definitive explanations await the isolation and characterization of the heretofore elusive apoptotic genes.

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