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Attenuation of glucocorticoid receptor levels by the H-*ras* oncogene

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Certain oncogene products are known to affect the cellular response to glucocorticoids. In particular, glucocorticoid-induced transcription is impaired in H-*ras*-transformed cells. In this study, we examine the mechanism for this effect in NIH3T3 cells containing stably integrated H-*ras* genomic sequences. NIH3T3*ras* cells transfected with the MMTV-CAT reporter exhibit a pronounced reduction in the level of glucocorticoid-induced CAT activity, compared to normal NIH3T3 cells. As the response to glucocorticoids depends on the amount of glucocorticoid receptor protein, we have examined the cellular receptor content in both cell lines. The cytosolic and total cellular GR protein are both markedly lower in NIH3T3*ras* cells, suggesting that the reduced response is directly due to an attenuation of receptor levels. The steady-state level of glucocorticoid receptor mRNA is appreciably reduced in NIH3T3*ras* cells, which accounts for the attenuated level of glucocorticoid receptor protein. The rate of glucocorticoid receptor gene transcription is concomitantly decreased in NIH3T3*ras* cells. The *ras* effect maps to the proximal promoter of the glucocorticoid receptor gene. These results suggest that a target for activated H-Ras protein may be a transcription factor which partially represses transcription of the glucocorticoid receptor gene.

Keywords: glucocorticoid receptor; H-*ras*; oncogene

Introduction

The cellular response to glucocorticoid agonists is mediated by the specific intracellular receptor for these ligands, the glucocorticoid receptor (GR). In the absence of hormone, the predominately cytoplasmic GR is one component of a hetero-oligomeric complex which also contains the heat shock proteins hsp90 and hsp56 (Catelli *et al.*, 1985; Housley *et al.*, 1985; Sanchez *et al.*, 1985; Tai *et al.*, 1986; Howard & Distelhorst, 1988; Sanchez, 1990; Rexin *et al.*, 1992). Upon ligand binding, the GR dissociates from this complex and undergoes a temperature-dependent transformation to the active, DNA-binding state (Mendel *et al.*, 1986; Sanchez *et al.*, 1987; Pratt, 1993). The GR translocates to the nucleus where it interacts with specific sequences in target genes to alter transcription (Yamamoto, 1985; Beato, 1989). The altered pattern of gene expression in response to glucocorticoids may be cell-type specific, depending on the particular target genes examined. Irrespective of the particular target gene, however, the magnitude of the glucocorticoid response is proportional to the cellular content of occupied GR (Bourgeois & Newby, 1979; Vanderbilt *et al.*, 1987).

Several studies have shown that certain oncogenes affect GR-mediated processes. One of the earliest reports suggesting that the glucocorticoid response is altered in cells expressing oncogene products demonstrated that infection of bone marrow preadipocytes with the Harvey, Kirsten, or Moloney strains of murine sarcoma virus blocks glucocorticoid-induced differentiation (Greenberger *et al.*, 1979). Expression of v-*mos* inhibits glucocorticoid induction of metallothionein (Hamilton & DeFranco, 1989; Touray *et al.*, 1991), and expression of activated H-*ras*, v-*mos*, or v-*src* causes a repression of glucocorticoid-dependent transcription from the mouse mammary tumor virus (MMTV) promoter (Jaggi *et al.*, 1986, 1988, 1989). Glucocorticoid induction of endogenous tyrosine aminotransferase (Jaggi *et al.*, 1989) and αB-crystallin (Aoyama *et al.*, 1993) is inhibited by H-*ras* and v-*mos* and glucocorticoid induction of glutamine synthetase is inhibited by H-*ras* (Martins & Brentani, 1990).

The *ras* gene family codes for small, guanine nucleotide-binding proteins that participate in a variety of cellular processes. Recent work by several groups has elucidated the *ras* signal transduction pathway initiated by extracellular mitogens (reviewed in Blenis, 1993; Crews & Erikson, 1993; Marx, 1993; McCormick, 1993). Activation of this signaling pathway by oncogenic H-*ras* or by mitogens such as platelet-derived growth factor or fibroblast growth factor results in the increased activity of a set of nuclear transcription factors including c-Myc, c-Jun and c-Fos (Müller *et al.*, 1984; Imler *et al.*, 1988; Schönthal *et al.*, 1988; Seth *et al.*, 1991). The activation of c-Jun and c-Fos by Ras appears to be mediated by distinct kinases in the cell (Deng & Karin, 1994; Westwick *et al.*, 1994). The transcription factor AP-1 is comprised of homodimers of c-Jun or heterodimers of c-Fos and c-Jun (Bohmann *et al.*, 1987; Angel *et al.*, 1988; Rauscher *et al.*, 1988; Sassone-Corsi *et al.*, 1988). Several groups have reported that a direct protein-protein interaction between the GR and AP-1 can occur *in vitro*, consistent with the proposal that there is a mutual inhibitory effect of AP-1 and GR on the transcription of certain genes (reviewed in Pfahl, 1993). The relative concentrations of c-Fos, c-Jun, and GR may also determine the degree of target gene expression. For example, Diamond *et al.* (1990) have shown that a single composite DNA element in the mouse proliferin gene behaves as either a stimulator or a repressor of GR-dependent transcription, depending on the relative amount of c-Jun or c-Fos.

Several studies from Groner's group have characterized the effects of H-*ras* and v-*mos* on glucocorticoid-dependent transcription (Jaggi *et al.*, 1986, 1988, 1989). In NIH3T3 cells containing stably integrated copies of these oncogenes under control of the MMTV-LTR promoter, glucocorticoid treatment results in the initial expression of MMTV-H-*ras* or MMTV-v-*mos*, followed by a decline in transcription from the MMTV promoter (Jaggi *et al.*, 1986, 1988). This repressive effect requires the protein product of the oncogene. Subsequent experiments using hormone-treated cells demonstrated that nuclear GR levels decline more rapidly in oncogene-transformed cells than their normal counterparts, suggesting that expression of H-*ras* or v-*mos* accelerates the rate of ligand-dependent down-regulation of the GR, perhaps by increasing the rate of GR protein degradation (Jaggi *et al.*, 1989). In light of the more recent observations that AP-1 interactions with the GR might reduce the level of glucocorticoid-dependent transcription, it seems reasonable to propose that increased levels of AP-1 in cells expressing the H-*ras* oncogene could account for an inhibition of GR-mediated transcriptional enhancement.

However, it seemed possible to us that an additional component in the mechanism of oncogene-mediated repression of

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GR-dependent transcription might be an alteration of GR gene expression. As this is difficult to evaluate in a cell system in which oncogene expression is itself dependent on ligand-bound GR transactivation, we have used NIH3T3 cells stably transfected with genomic sequences containing the promoter and structural gene for activated H-*ras*(val12) to examine this possibility. In an earlier report, Martins and Brentani (1990) found that glucocorticoid induction of glutamine synthetase was markedly impaired in NIH3T3*ras* cells, compared to normal NIH3T3 cells. The GR in NIH3T3*ras* cells is apparently normal with respect to ligand affinity, M_r, thermostability, and transformation to the DNA-binding state. However, a reduced level of cytosolic glucocorticoid specific binding capacity was observed in NIH3T3*ras* cells. In the present paper, we have examined the basis for the reduced glucocorticoid response in NIH3T3*ras* cells. We find that the total cellular GR protein and GR mRNA levels are markedly reduced in NIH3T3*ras* cells, compared to normal NIH3T3 cells. This reduction is due, at least in part, to an attenuated transcription rate of the GR gene. The oncogene effect maps to the proximal promoter of the GR gene, suggesting that one cellular target for the H-*ras* $p21^{val12}$ protein is a nuclear transcription factor which acts directly or indirectly at these promoter sequences to partially repress GR gene transcription.

Results

Glucocorticoid-inducible CAT activity is attenuated in ras-*transformed cells*

We have previously demonstrated that glucocorticoid induction of glutamine synthetase is defective in NIH3T3*ras* cells (Martins & Brentani, 1990). As the magnitude of this response is rather modest (approximately two-fold) in normal NIH3T3 cells, we wanted to compare the glucocorticoid response in these cell lines using a more sensitive assay. Accordingly, we transfected NIH3T3 and NIH3T3*ras* cells with the glucocorticoid-inducible reporter plasmid MMTV-CAT, which contains the bacterial chloramphenicol acetyltransferase (CAT) gene under control of the MMTV promoter (Danielsen *et al.*, 1986). As shown in Figure 1, dexamethasone elicited almost a nine-fold induction of CAT activity in transfected NIH3T3 cells. By contrast, in NIH3T3*ras* cells CAT activity was only induced two-fold, consistent with the reduced induction of glutamine synthetase activity previously observed in these cells (Martins & Brentani, 1990). This reduction in CAT activity is not due to a difference in the relative stability of the CAT enzyme in NIH3T3*ras* cells, as both cell lines exhibited the same level of CAT activity when transfected with the constitutive reporter plasmid SV2CAT (Figure 1). The simplest interpretation is that NIH3T3*ras* cells contain lower amounts of active GR. This is partially confirmed by the observation that cotransfection with MMTV-CAT and a mouse GR cDNA expression vector results in a marked increase in the level of dexamethasone-induced CAT activity for NIH3T3*ras* cells (Figure 1). In an earlier report, we found that the cytosolic specific glucocorticoid binding capacity was reduced in NIH3T3*ras* cells (Martins & Brentani, 1990). We also assayed the GR in cell-free extracts over the course of the present studies. The specific glucocorticoid binding capacity in cytosol preparations from parent NIH3T3 cells (1.8 pmol/mg protein) was markedly higher than that in cytosol from NIH3T3*ras* cells (0.3 pmol/mg protein).

*Total immunoreactive GR protein levels are reduced in NIH3T3*ras *cells*

The value for glucocorticoid specific binding capacity in each cell line is a measure of the GR associated with hsp90 in an untransformed heterocomplex (Bresnick *et al.*, 1989; Picard

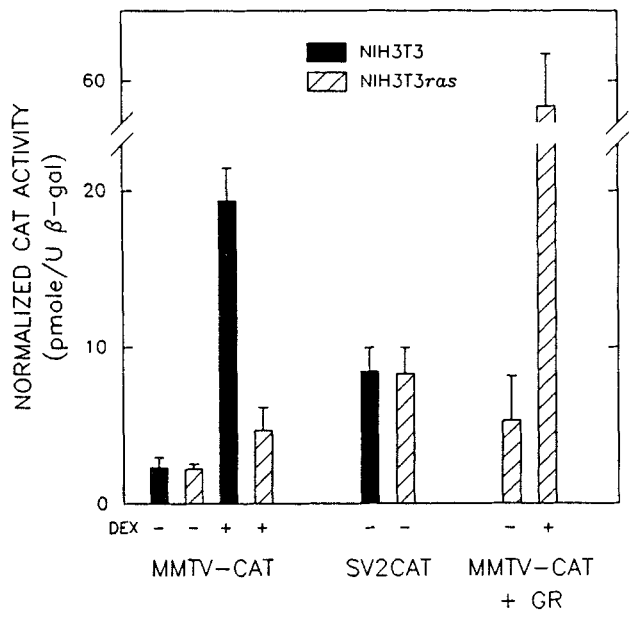


Figure 1 Glucocorticoid induction of MMTV-CAT in NIH3T3 and NIH3T3*ras* cells. NIH3T3 and NIH3T3*ras* cells were transfected with MMTV-CAT, SV2CAT or the GR expression vector as indicated. CMVβgal was included in each transfection as an internal control. Cell extracts were prepared 72 h later and assayed for CAT and β-galactosidase activities. For cells transfected with MMTV-CAT, dexamethasone (1 μM) or vehicle was added 16 h before harvesting. CAT activity in each case is normalized for β-galactosidase activity in the same extract (mean ± S.D., $n = 3$)

et al., 1990; Scherrer *et al.*, 1990). It seemed possible that one effect of activated H-*ras* expression might be an alteration in the assembly of GR complexes with hsp90 and other components. Thus, the amount of active GR might be lowered without changing the total GR content. In order to determine if total GR protein was decreased in NIH3T3*ras* cells, the amounts of immunoreactive GR protein and receptor-associated hsp90 protein were analysed in NIH3T3 and NIH3T3*ras* cells using quantitative immunoblotting. Figure 2A shows that the ~100 kDa band corresponding to the mouse GR was markedly reduced in cytosol from NIH3T3*ras* cells, compared to normal NIH3T3 cells. Assay of bound ^{125}I-labeled antibody determined that NIH3T3*ras* cells contain approximately 20% the level of cytosolic GR present in normal cells. The amount of GR-associated hsp90 in each sample was estimated by the ratio of hsp90:GR radioactivity. Identical values were obtained, suggesting that there is no appreciable difference in GR complex formation in NIH3T3*ras* cells. We also compared total GR protein in whole cell extracts and in nuclear extracts of each cell line to determine if there was a difference in the subcellular distribution of unliganded GR (Figure 2B). Compared to normal NIH3T3 cells, NIH3T3*ras* cells contained 26% the level of total immunoreactive GR protein. The GR is predominately cytosolic in the absence of hormone treatment, so relatively large amounts of nuclear extract protein were analysed for GR protein (Figure 2B). The small amount of nuclear GR in NIH3T3*ras* cells was approximately 80% of the amount in normal cell nuclei, which indicates that there is no marked difference in the subcellular distribution of GR in H-*ras*-transformed cells. Thus, we conclude that the reduced response to glucocorticoid induction of glutamine synthetase (Martins & Brentani, 1990) and MMTV-CAT (Figure 1) in NIH3T3*ras* cells is due to a marked reduction in the total cellular level of GR protein.

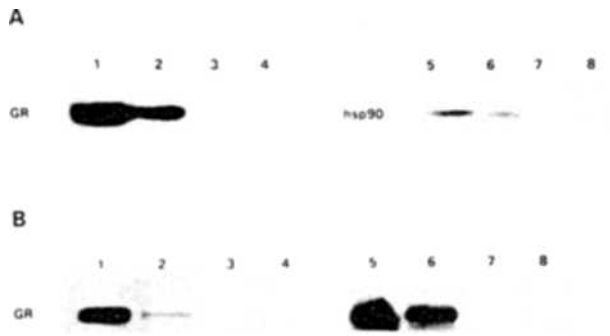


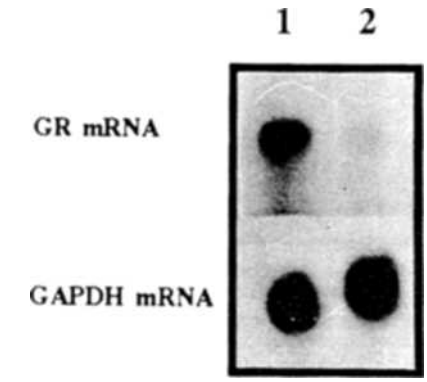
Figure 2 Immunoblot analysis of glucocorticoid receptor protein in NIH3T3 and NIH3T3*ras* Cells. (**A**) Aliquots of cytosol prepared from NIH3T3 cells (lanes 1, 3, 5,and 7) or NIH3T3*ras* cells (lanes 2, 4, 6 and 8) containing 7 mg protein were immunoadsorbed with BuGR2 anti-receptor antibody (lanes 1, 2, 5 and 6) or normal mouse IgG (lanes 3, 4, 7 and 8), resolved by SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon P. The blot was incubated with BuGR2 (lanes 1–4) or AC88 anti-hsp90 antibody (lanes 5–8) followed by ^{125}I-labeled anti-mouse IgG. GR and hsp90 immunoreactive proteins were visualized by autoradiography of the blot. The autoradiogram shown was overexposed with respect to the GR in order to reveal hsp90. (**B**) Aliquots of whole cell extract containing 12 mg protein (lanes 1–4) or of nuclear extract containing 40 mg protein (lanes 5–8) from NIH3T3 cells (lanes 1, 3, 5, and 7) or NIH3T3*ras* cells (lanes 2, 4, 6 and 8) were immunoadsorbed with BuGR2 (lanes 1, 2, 5 and 6) or normal mouse IgG (lanes 3, 4, 7, and 8), resolved by SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon P. The blot was incubated with BuGR2 followed by ^{125}I-labeled anti-mouse IgG. GR immunoreactive protein was visualized by autoradiography

The steady-state GR mRNA level is attenuated in ras-*transformed cells*

As NIH3T3*ras* cells contain less GR protein than non-transformed NIH3T3 cells, we wanted to determine if there was a corresponding difference in the amount of GR mRNA. Total cellular RNA was isolated from both cell lines and probed for GR mRNA. The upper portion of Figure 3 shows a representative RNA blot from these experiments. NIH3T3 and NIH3T3*ras* cells contained a single GR mRNA of approximately 7 kb, similar to other murine cell lines (Lucas *et al.*, 1988; Housley & Forsthoefel, 1989). Hybridization with the GAPDH probe confirmed that equal amounts of RNA were loaded in each lane. The results from four RNA blots are shown in the lower part of Figure 3. The GR mRNA level in NIH3T3*ras* cells was 27% of the level present in normal NIH3T3 cells. Therefore, the lower GR protein and specific binding capacity values in NIH3T3*ras* cells are due, at least in part, to a reduction in the amount of GR mRNA.

It is important to note that this reduction is not limited to this particular clone of NIH3T3*ras* cells, nor is it a consequence of long-term culture of the NIH3T3*ras* cells. In order to exclude clone-specific effects, we stably transfected NIH3T3 cells with the activated H-*ras* expression vector. The resulting clones (termed RARG cells) which exhibited a transformed morphology were expanded and assayed for glucocorticoid specific binding capacity and GR mRNA content. As shown in Figure 4, ten representative clonal RARG cell lines exhibited a reduction in GR mRNA. This reduction varied from 50–80%, and was correlated with a concomitant reduction in glucocorticoid specific binding of 42–77%.

In order to test if the H-*ras* effect on GR levels was limited to NIH3T3 cells, we also transfected the activated H-*ras* construct into another well-characterized murine fibroblast cell line, L929 cells. Stably transfected LRAS clones were isolated and the GR mRNA level and specific binding values were determined. As shown in Figure 5, the presence of the



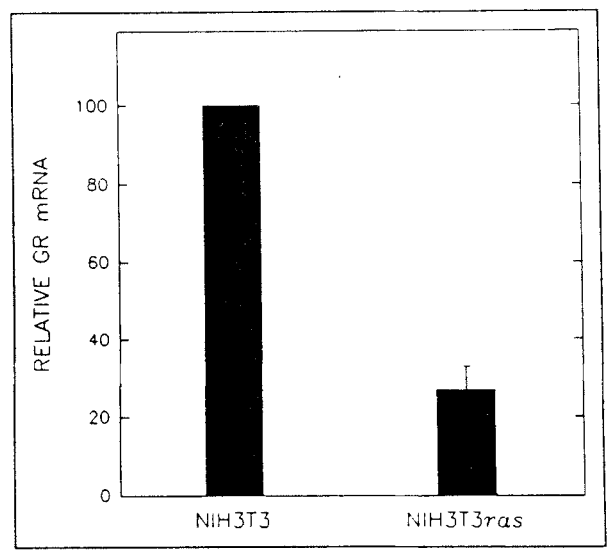


Figure 3 The level of glucocorticoid receptor mRNA is reduced in NIH3T3*ras* cells. Total RNA was isolated from NIH3T3 cells (lane 1) or NIH3T3*ras* cells (lane 2), fractionated on a formaldehyde-agarose gel, and blotted to a nylon filter. The blotted RNA was hybridized successively with mouse GR and GAPDH ^{32}P-labeled cDNA probes, and the specific RNA transcripts were visualized by autoradiography. GR mRNA levels from four independent RNA blots were quantified using scanning densitometry. The level of GR mRNA in NIH3T3*ras* cells, normalized to the internal reference signal, is shown relative to the value obtained from NIH3T3 cells (mean ± S.D., $n = 4$)

activated H-*ras* oncogene in these LRAS cells reduced the level of GR mRNA by 50–86%, with a similar range of reduced specific binding capacity (40–69%) compared to parent L929 cells.

We also analysed genomic GR DNA sequences to determine if the reduced GR mRNA in NIH3T3*ras* cells might be due to insertion of H-*ras* sequences near the 5′-end of the GR coding sequences. Digestion of genomic DNA with four restriction enzymes revealed no differences in genomic GR sequences using an N-terminal fragment of the mouse GR cDNA as the probe (data not shown), suggesting that there has been no substantial rearrangement of GR sequences due to insertion of H-*ras* DNA.

The transcription rate of the GR gene is decreased in ras-*transformed cells*

The steady-state level of a particular mRNA depends on both the rates of synthesis and degradation. In order to determine whether the attenuated steady-state GR mRNA

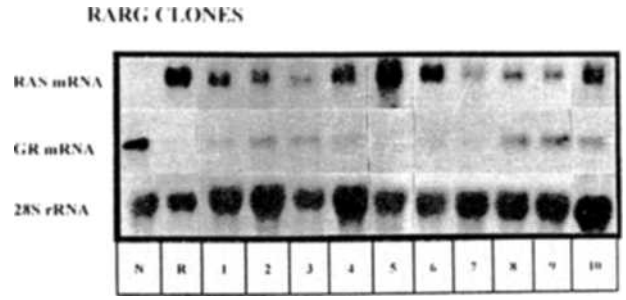


Figure 4 Glucocorticoid receptor mRNA levels are lower in RARG cells. Total cellular RNA was isolated from normal parent NIH3T3 cells (**N**), NIH3T3*ras* cells (**R**), and ten RARG clonal cell lines derived from transfection of NIH3T3 cells with the activated H-*ras* gene. After size fractionation on denaturing gels and transfer to nylon membranes, RNA samples were probed for H-*ras* mRNA, GR mRNA, and 28*S* rRNA. Following autoradiography, the specific bands were quantified using scanning densitometry

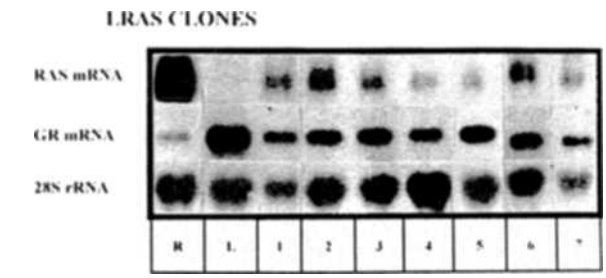


Figure 5 Glucocorticoid receptor mRNA levels are lower in H-*ras*-transformed L929 cells. Total cellular RNA was isolated from NIH3T3*ras* cells (**R**), parent L929 cells (**L**), and seven LRAS clonal cell lines derived from transfection of L929 cells with the activated H-*ras* gene. RNA samples were analysed as described in the legend to Figure 4

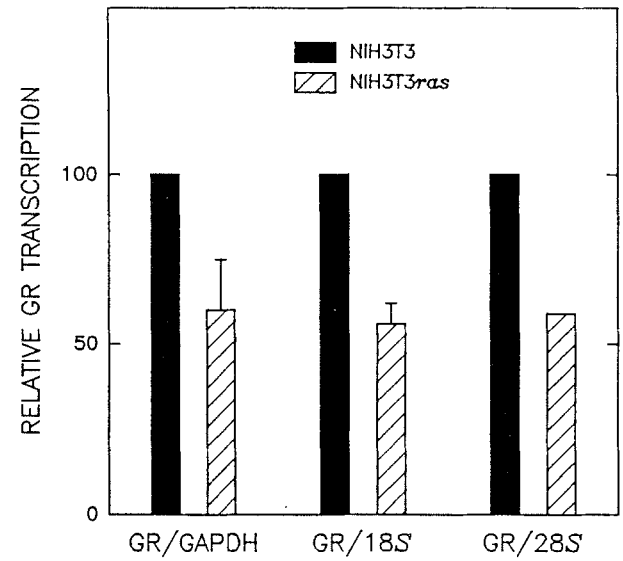


Figure 6 Nuclear run-on analysis of glucocorticoid receptor gene transcription. Radiolabeled RNA was synthesized in nuclei from NIH3T3 and NIH3T3*ras* cells, purified, and hybridized to immobilized target DNA sequences. The value obtained for GR mRNA is normalized to each internal control (GADPH mRNA, 18*S* rRNA, or 28*S* rRNA) and is depicted as the level relative to normal NIH3T3 cells in each case. The results represent the mean value (±S.D.) from two independent experiments using GAPDH and 18*S* rRNA, and the value obtained from one experiment using 28*S* rRNA

level in NIH3T3*ras* cells is due to a decreased transcriptional rate, nuclear run-on transcription experiments were performed using nuclei isolated from each cell line. Figure 6 shows the relative GR gene transcription in each cell line using GAPDH mRNA, 18*S* rRNA, and 28*S* rRNA as internal controls. Normalized GR gene transcription in NIH3T3*ras* cells was approximately 60% of the value observed in NIH3T3 cells, regardless of the internal control used.

Thus, reduced GR gene transcription in H-*ras*-transformed cells accounts for the reduction in GR mRNA and protein levels in these cells.

*GR gene promoter activity is decreased in NIH3T3*ras *cells*

Among the many cellular effects of activated Ras protein is an altered pattern of gene expression, particularly in growth-regulating genes. A change in the expression of a particular gene occurs as a result of the efficiency of transcription initiation at the promoter, which results from the combined actions of multiple transcription factors. In order to determine if H-*ras* transformation of NIH3T3 cells results in altered GR gene promoter activity, we transfected NIH3T3*ras* cells and parent NIH3T3 cells with a CAT gene reporter under the control of human GR gene promoter sequences (Zong *et al.*, 1990). This promoter region corresponds to the mouse GR promoter designated P1C (Strahle *et al.*, 1992). In transfected NIH3T3*ras* cells, the resulting CAT activity was less than half the value obtained in normal cells (Figure 7). As we have shown that CAT expression under control of the constitutive SV40 early promoter is identical in each of these cell lines (Figure 1), this result strongly suggests that one consequence of H-*ras* oncogene expression is to partially repress transcription from the proximal promoter of the GR gene. This conclusion is further supported by the observation that when the H-*ras* expression vector was cotransfected with the GR promoter-CAT construct into normal NIH3T3 cells, the level of CAT expression was reduced by approximately one-half (Figure 7). This conclusion is also strengthened by the results of nuclear run-on assays (Figure 6), which demonstrated a reduction in the rate of endogenous GR gene transcription in NIH3T3*ras* cells.

Discussion

We have previously reported that glucocorticoid-induced glutamine synthetase activity was markedly reduced in H-*ras*-transformed NIH3T3 cells compared to normal cells (Martins & Brentani, 1990). In the present work, we have shown that NIH3T3*ras* cells also exhibit an attenuated response after transfection with the glucocorticoid-dependent MMTV-CAT reporter plasmid. The level of glucocorticoid specific binding capacity is appreciably reduced in NIH3T3*ras* cells, suggesting that either GR complex assembly with hsp90 is defective or that the cellular GR content is reduced. As the total GR protein and GR mRNA are decreased considerably in NIH3T3*ras* cells, and as there is no difference in the ratio of receptor-associated hsp90 in these two cell lines, we propose that the altered steroid response in these cells is directly due to the reduction in the cellular GR level. This proposal is consistent with reports demonstrating that cellular GR content is roughly proportional to the glucocorticoid response (Bourgeois & Newby, 1979; Vanderbilt *et al.*, 1987). A difference in steady-state mRNA levels could be attributed to altered rates of synthesis, degradation, or both. We have examined the apparent transcription rate of the GR gene in both cell lines and we find a 40% decrease in NIH3T3*ras* cells. Our experiments with the GR promoter-CAT construct

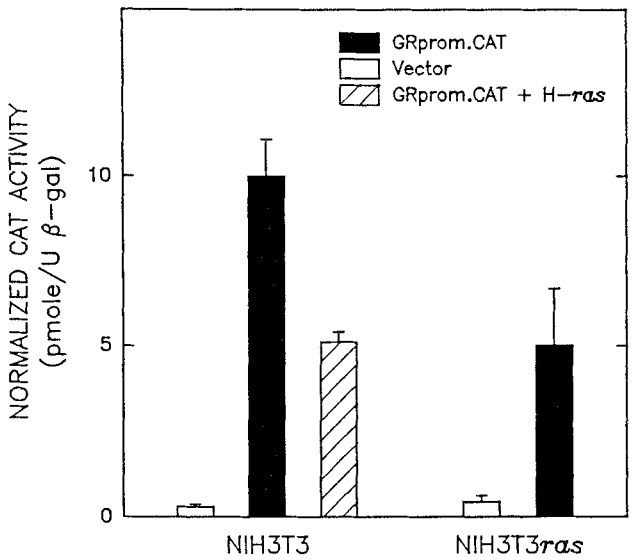


Figure 7 Glucocorticoid receptor promoter activity is reduced in NIH3T3*ras* cells. NIH3T3 and NIH3T3*ras* cells were cotransfected with CMVβgal and either the promotorless vector, the GR promoter-CAT construct, or GR promoter-CAT plus the activated H-*ras*(val12) expression vector as shown. After 72 h, cell extracts were prepared and assayed for CAT and β-galactosidase activity. CAT activity was normalized to β-galactosidase activity in each experiment (mean ± S.D., $n = 3$)

demonstrate that the Ras effect maps to sequences contained within the 2.7 kb fragment of genomic DNA proximal to the second, translated exon of the GR gene. While we cannot exclude the possibility that H-*ras* oncogene expression also results in an increased degradation of GR protein in the absence of hormone, it seems likely that the reduced level of GR in these cells is due predominately to a corresponding reduction in the transcription rate of the GR gene. As we have not yet tested other types of cells, such as those of hepatic or lymphocyte origin, it remains to be determined if the effect of activated H-Ras on GR expression is restricted to fibroblasts. However, this effect is clearly not limited to the particular clone of NIH3T3*ras* cells characterized in this work, as we have observed a similar reduction of GR mRNA in several independent isolates of NIH3T3 and L929 cells after stable transfection with activated H-*ras* (Figures 4 and 5).

The effects of the H-*ras* oncogene on GR-mediated transcription have been examined previously by several groups. Jaggi *et al.* (1986, 1988, 1989) have shown that cells stably transfected with MMTV-H-*ras* respond to dexamethasone by an initial increase in H-*ras* expression, followed by a decline. This repression of transcription from the MMTV promoter requires the H-Ras protein and is accompanied by a decline in nuclear GR protein levels, compared to cells which do not contain activated H-Ras. This result suggested that the presence of activated H-Ras predisposes cells to increase the rate of ligand-dependent GR down-regulation, probably through increased degradation of GR protein (Jaggi *et al.*, 1989). Consistent with this proposal, in NIH3T3 cells stably transfected with the genomic H-*ras* oncogene under control of the H-*ras* promoter, there is a decrease in glucocorticoid-dependent expression of glutamine synthetase (Martins & Brentani, 1990), MMTV-CAT (Vacca *et al.*, 1989) and αB-crystallin (Aoyama *et al.*, 1993). However, the present results suggest that a major effect of H-*ras* is the reduced expression of the GR gene, even in the absence of hormone.

As one effect of H-*ras* oncogene expression is an increased level of AP-1 activity, it is appropriate to consider how AP-1 affects GR-mediated transcription. Overexpression of c-Fos or c-Jun in cells transfected with glucocorticoid-inducible CAT constructs results in a decrease in the hormone-induced level of CAT activity. This effect was observed in NIH3T3 cells (Jonat *et al.*, 1990; Lucibello *et al.*, 1990; Schule *et al.*, 1990) and HeLa cells (Lucibello *et al.*, 1990; Yang-Yen *et al.*, 1990). In these papers a model was proposed for mutually repressive interactions between GR and transcription factor AP-1. This potential regulatory mechanism does not require receptor-DNA interactions, but rather protein-protein interaction between the steroid receptor and AP-1, resulting in biological antagonism (Pfahl, 1993). However, the experiments that led to this model did not examine the possibility that AP-1 could also have separate effects on GR gene expression. The influence of AP-1 components on transcriptional activation by steroid receptors has been more extensively examined by Shemshedini *et al.* (1991). Using multiple reporter genes and cell lines, they found that c-Fos inhibits and c-Jun either inhibits or stimulates progestin, glucocorticoid, and androgen receptor-induced transcription. The effects were receptor, promoter, and cell type specific, and the steroid receptors had non-reciprocal effects on the transactivation ability of c-Jun. Maroder *et al.* (1993) have assessed the effects of AP-1 on GR-dependent transcription in several lymphoid cell lines, HeLa cells, and NIH3T3 cells transfected with MMTV-CAT and c-*jun*/c-*fos* expression vectors. These authors conclude that there is a cell-specific bifunctional role of c-Jun and a negative inhibitory role of c-Fos on GR-dependent transcription. These observations suggest that abortive heterodimer formation between AP-1 and steroid receptors, resulting in mutual inhibition of DNA binding, cannot fully account for the effects of AP-1 on steroid receptor transactivation (Shemshedini *et al.*, 1991; Maroder *et al.*, 1993). The possibility that AP-1 has direct effects on GR gene expression is compatible with these conclusions, and it can be tested using hormone-free cells in which the extranuclear GR is unable to participate in direct protein-protein interactions with AP-1.

Expression of the mouse GR gene has been shown to be controlled by at least three distinct promoters, and one promoter is cell-specific for T lymphocytes (Strahle *et al.*, 1992). The human GR promoter sequence used in our experiments corresponds to the proximal P1C promoter in murine DNA, which is also the strongest promoter in fibroblasts (Strahle *et al.*, 1992). Although GR gene expression is complex and not yet clearly defined, our results suggest that expression of the H-*ras* oncogene in fibroblasts somehow alters the complex of transcription factors that facilitate initiation of RNA synthesis at the proximal GR promoter. The GR promoter lacks TATA or CAAT elements but it contains several GC boxes corresponding to binding sites for transcription factor Sp1 (Zong *et al.*, 1990; Enrico & Detera-Wadleigh, 1991; Leclerc *et al.*, 1991). There are also several regions with similarity to consensus binding sites for AP-1, NF-1, and CREB although the functional significance of these sites has not been established.

There are a number of potential mechanisms by which the H-*ras* oncogene might affect GR expression. One possibility is that activated H-Ras results in deregulation of the level of a particular nuclear transcription factor which binds directly to the GR promoter and represses transcription. A related possibility is that the deregulated transcription factor acts indirectly by inducing the synthesis of a protein which represses GR gene transcription. Either model is consistent with the known effects of H-*ras* on cells and with the observations reported in this paper. There are also other nuclear proteins which are affected by *ras* oncogenes. Owen *et al.* (1990) have shown that there is a functional *ras* responsive enhancer DNA element which is required for *ras* effects on expression of the NVL-3 and TGF-β genes. Kedar *et al.* (1990) have demonstrated a *ras*-responsive element in the DNA β-polymerase promoter. A 120 kDa nuclear protein present in cells expressing activated *ras* genes binds to the *ras*

response element and probably mediates some of the cellular effects of *ras* oncogenes (Owen & Ostrowski, 1990a,b). The K-*ras* oncogene activates the promoter of the murine intracisternal A particle gene through a potential cAMP response element (Galien *et al.*, 1991), suggesting that a CREB-like protein participates in certain cellular responses to *ras* oncogenes.

Presently, we favor a model in which GR gene expression is attenuated by H-*ras*-mediated overexpression of one or more nuclear transcription factors. Implicit to this model is the prediction that at points in the cell cycle when the level of this putative protein is elevated, there should be a corresponding decrease in the rate of GR gene transcription. Conversely, when the transcription rate of the GR gene is maximal, there should be a concomitant minimal amount of this transcription factor. As cellular GR levels have been shown to vary during the cell cycle (Cidlowski & Cidlowski, 1982), and as c-Myc, c-Fos, and c-Jun are synthesized in a transient manner and have been shown to be altered in their levels by activated H-*ras*, it seems reasonable to propose that one or more of these proteins acts in a negative manner on the rate of GR gene transcription. This idea is supported by the recent observation that overexpression of c-Jun represses CAT gene expression in cells transfected with the GR promoter-CAT construction (Martins *et al.*, 1994). We are currently examining the expression of the genes coding for AP-1 proteins in H-*ras*-transformed cells and determining how overexpression of these proteins affects GR gene expression.

Materials and methods

Materials

[6,7-^{3}H]Dexamethasone (48.2 Ci/mmole), [^{125}I]goat anti-mouse IgG (5.9 μCi/μg), and deoxycytidine 5′-[α-^{32}P] triphosphate (3000 Ci/mmole) were from New England Nuclear. Uridine 5′-[α-^{32}P] triphosphate (650 Ci/mmol) and [^{3}H]acetate (66 Ci/mmole) were from ICN. Protein A-Sepharose, radioinert dexamethasone, S-Acetyl Coenzyme A synthetase, and Coenzyme A were from Sigma Chemical Co. The BuGR2 anti-receptor monoclonal antibody (Gametchu & Harrison, 1984) was provided by Drs. William J. Hendry and Robert W. Harrison. The AC88 anti-hsp90 monoclonal antibody (Riehl *et al.*, 1985) was provided by Dr. David O. Toft. The mouse GR cDNA expression plasmid SV2Wrec and the glucocorticoid-inducible MMTV-CAT reporter plasmid (Danielsen *et al.*, 1986) were obtained from Drs. Mark Danielsen, Jeffrey P. Northrop, and Gordon M. Ringold. The plasmid CMVβgal containing the *E. coli* β-galactosidase gene under control of the immediate early cytomegalovirus promoter (MacGregor & Caskey, 1989) was provided by Dr. Grant R. MacGregor. The p11a-2 plasmid containing the 28S rRNA insert (Wahl *et al.*, 1983) was provided by Dr. Geoffrey M. Wahl. The pGPDN5 plasmid containing the rat glyceraldehyde-3-phosphate dehydrogenase cDNA (Fort *et al.*, 1985) was provided by Dr. Ph. Fort. The H-*ras* expression vector pSVET24, coding for H-Ras (val12) protein (Capon *et al.*, 1983), was provided by Dr. B. Fennie. The pCAT-Enhancer plasmid and RNAsin were obtained from Promega. The GR promoter-CAT plasmid, provided by Dr. E. Brad Thompson and Jing Zong, contains 2.7 kb of the human GR promoter sequence (Zong *et al.*, 1990) cloned into the SalI site of the pCAT-Enhancer plasmid.

Cell culture and extract preparation

Parent NIH3T3 cells and NIH3T3*ras* cells, stably transformed with the activated H-*ras* oncogene (Capon *et al.*, 1983), were provided by Dr. Bernd Groner. Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone). All subsequent steps were performed at 4°C. Cells were suspended in 1.5 volumes of 10 mM Hepes, pH 7.35, 1 mM EDTA, 20 mM sodium molybdate and ruptured in a Dounce homogenizer. Nuclei were recovered by centrifugation at 600 *g* for 15 min. The supernatant fluid was further centrifuged at 105 000 *g* for 1 h to recover the soluble cytosol fraction. Specific glucocorticoid binding capacity was assayed in cytosol as described (Housley, 1990). Nuclear pellets were washed and incubated in extraction buffer (10 mM sodium phosphate pH 7.35, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 0.5 mM benzamidine, 0.5 mM PMSF, 5 μg/ml leupeptin) for 20 min and centrifuged at 105 000 *g* for 1 h to obtain the soluble nuclear extract. Whole cell extracts were obtained after incubating cells in extraction buffer for 20 min and centrifuging at 100 000 *g* for 1 h.

Immunoadsorption, gel electrophoresis and immunoblotting

Aliquots of cytosol, nuclear extract, or whole cell extract were diluted with an equal volume of 10 mM TES, pH 7.6, 50 mM NaCl, 10% glycerol, 4 mM EDTA, 20 mM sodium molybdate and incubated with BuGR2 antibody or normal mouse IgG before adsorption to protein A-Sepharose as previously described (Housley, 1990). Immunoadsorbed proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon P membranes (Millipore). GR and hsp90 proteins were detected using the BuGR2 and AC88 antibodies, respectively, and quantified as previously described (Housley, 1990). Briefly, after probing with the primary antibodies the immune complexes were detected by incubation with ^{125}I-labeled goat anti-mouse IgG and peroxidase-conjugated anti-goat IgG. After peroxidase staining and autoradiography, stained bands were excised and assayed for [^{125}I] by γ crystal scintillation spectrometry.

Cell transfection and enzyme assays

Cells growing in 162 cm^2 flasks were transiently transfected using the calcium phosphate technique with 20 μg of the particular CAT plasmid (MMTV-CAT, SV2CAT, pCAT-Enhancer, or GR promoter-CAT) plus 20 μg of CMVβgal DNA. For some experiments, the GR and H-*ras* expression plasmids were also included. Cells were harvested 72 h after transfection and extracts prepared by freeze/thaw lysis in 250 mM Tris pH 7.6. For induction of MMTV-CAT expression, 1 μM dexamethasone was added 16 h before cell harvest. A portion of each extract was saved for β-galactosidase determination (Sambrook *et al.*, 1989) and 5 mM EDTA was added to the remainder before heating at 65°C (Crabb & Dixon, 1987). Heated extracts were assayed for CAT activity as described by Nordeen *et al.* (1987). CAT activity was normalized to units of β-galactosidase activity to correct for minor differences in transfection efficiency among experiments. NIH3T3 and L929 cells were stably transfected using the calcium phosphate technique with the pSVET24 and SV2neo plasmids. The pSVET24 plasmid contains the genomic coding sequences of the activated human H-*ras* (val12) gene under control of the SV40 early promoter (Capon *et al.*, 1983). After selection in medium containing 0.5 mg/ml G418, clones exhibiting transformed morphology were isolated. Cloned stable transfectants derived from NIH3T3 cells were termed RARG cells, and those from L929 cells were termed LRAS cells.

RNA blot analysis

Total cellular RNA from cultured cells was isolated as described by Yasumoto *et al.* (1987). Samples containing 20 μg of formamide-denatured RNA were fractionated on formaldehyde-agarose gels, transferred to a Hybond nylon membrane (Amersham), and hybridized to radiolabeled cDNA probes as previously described (Housley & Forsthoefel, 1989). The 1.0 kb HindIII fragment of pSV2Wrec, the 1.2 kb

PstI fragment of pGPDN5, and the complete inserts of pSVET24 and p11a-2 were radiolabeled with [α-^{32}P]dCTP by random priming and used as probes for GR mRNA, GAPDH mRNA, H-*ras* mRNA and 28*S* rRNA, respectively. The signal intensity of each autoradiographic band was quantified using scanning laser densitometry. GR and H-*ras* mRNA signals were normalized to GAPDH mRNA or 28*S* rRNA signals in the same samples.

Nuclear run-on transcription analysis

For preparation of nuclei, cells were harvested and washed with ice-cold phosphate-buffered saline, pelleted by centrifugation and resuspended in 5 ml ice-cold NP-40 lysis buffer (15 mM HEPES pH 7.5, 300 mM sucrose, 15 mM NaCl, 60 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol and 0.1% NP-40). After a 5 min incubation on ice, cell lysates were centrifuged at 500 *g* for 5 min at 4°C. The nuclear pellets were suspended in 1 ml glycerol storage buffer (Woodworth *et al.*, 1990), washed twice in this buffer, and used immediately. Nuclear run-on analyses were performed as previously described (Woodworth *et al.*, 1990). The transcription reaction was terminated by the addition of 4 ml GTC solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sodium sarkosyl, 0.1 M β-mercaptoethanol) and RNA was purified by centrifugation through cesium-trifluoroacetate gradients as previously described (Yasumoto *et al.*, 1987). Equal amounts of radiolabeled RNA were hybridized to excess target DNAs immobilized on Hybond N-plus membranes (Amersham) for 72 h at 42°C. Each membrane contained 25 μg of each of the following plasmids: pSV2Wrec, pGPDN5, p11a-2, pBR322, pSV2CAT and pBR322 containing 1.9 kb of the mouse 18S rRNA gene (Arnheim, 1979). Membranes were washed at room temperature and at 42°C with 2× SSPE containing 0.5% SDS. After autoradiography, each localized band was excised and the amount of hybridized RNA was quantified by liquid scintillation spectrometry. Specific hybridization was calculated by subtracting the radioactivity bound to vector DNA sequences. The normalized GR gene transcription represents the ratio of the specific GR mRNA radioactivity to the specific radioactivity of the internal controls for GAPDH mRNA, 28*S* rRNA, or 18*S* rRNA.

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