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IN VIVO INHIBITION OF GLUCOCORTICOID-INDUCIBLE GENE EXPRESSION BY DIMETHYLNITROSAMINE IN RAT LIVER

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Abstract—Sprague–Dawley rats were pretreated with a single i.p. injection of either 2.25 mL/kg of phosphate-buffered saline (PBS) or 22.5 mg/kg of dimethylnitrosamine (DMN) followed 2 hr later by a single i.p. injection of either 1.35 mg/kg of dexamethasone (DEX) or the vehicle, a 50% ethanol solution, both delivered in a volume of 3 mL/kg. RNA levels of the hormone-inducible, specialized liver function genes, tyrosine aminotransferase (TAT) and glutamine synthetase (GS), were monitored 4, 5, 6, 7, 8, and 10 hr after the DEX injection. Maximal induction of both the TAT (26-fold) and GS (6-fold) RNAs occurred 6 hr after DEX administration in PBS-pretreated animals. Pretreatment with DMN caused at least a 42% inhibition of DEX-induced RNA accumulation at every time point examined, with greater than 90% inhibition occurring when the genes were maximally induced at 6 hr. This inhibition was not due to any alterations of the glucocorticoid receptors as DMN had no effect on the binding affinity or amounts of glucocorticoid receptors present in rat hepatic cytosols. These results suggest that chemical carcinogens such as DMN may affect normal gene function *in vivo* by inhibiting the cellular response to hormone receptors mediating differentiation-associated, specialized cell functions.

Recent studies in molecular oncology have focused on alterations in the expression and function of protooncogenes, such as growth factors and G-proteins, and tumor suppressor genes, such as p53 and the retinoblastoma gene, in an attempt to gain a mechanistic understanding of the alterations in growth regulatory pathways that mediate the transformation of normal cells to cancer cells. However, in addition to these important changes in the expression and function of growth regulatory genes, neoplastic transformation is usually accompanied by the down-regulation of specialized function genes normally associated with the differentiated phenotype of the cell. This laboratory has been using glucocorticoid-sensitive genes as a model system in which to study the effects of chemical carcinogens on normal cellular gene expression. Steroids have proven to be quite amenable to mechanistic studies at the molecular level, thus their induction of gene expression is probably the best characterized eukaryotic gene regulatory system [1, 2].

Since steroid-sensitive genes often code for organ-specific enzymes involved in specialized cell functions, these gene products have been used as markers for the alterations of cellular differentiation that occur upon neoplastic transformation. Studies in rat and human hepatoma cell lines have shown that cell clones exhibiting a more differentiated phenotype have higher levels of expression of their specialized function genes, whereas those clones exhibiting a less differentiated phenotype often lose their cell-specific gene functions and inappropriately express fetal-specific gene products [3–6]. Among those genes shown to be down-regulated in the less differentiated clones was the tyrosine aminotransferase (TAT§) gene, a specialized liver function enzyme involved in gluconeogenesis.

TAT enzyme activity was decreased markedly in the late stages of azo dye-induced hepatocarcinogenesis in the rat [7], in good agreement with an earlier study demonstrating an azo dye-mediated inhibition of steroid-induced TAT activity in fetal rat hepatocytes [8]. Treatment of rats *in vivo* with either aflatoxin B_1 (AFB) [9] or dimethylnitrosamine (DMN) [10] inhibited the glucocorticoid-mediated induction of TAT [9] and tryptophan pyrrolase [9, 10] enzyme activity. Since AFB inhibited TAT activity in a manner analogous to actinomycin D, it was suggested that the carcinogen acted at a pretranslational step to prevent protein synthesis. Later studies by Kensler *et al.* [11, 12] provided further evidence for a pretranslational site of carcinogen action. Using *in vitro* binding studies to measure formation of the steroid hormone–receptor complex and binding of this complex to nuclear DNA, the authors demonstrated that while chemical carcinogens had no effect on the K_d and B_{max} of steroid binding to its receptor, both the K_d and the number of nuclear acceptor sites were decreased when dexamethasone

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§ Abbreviations: AFB, aflatoxin B_1; DEX, dexamethasone; DMN, dimethylnitrosamine; GCR, glucocorticoid receptor; GS, glutamine synthetase; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; 1× SSC, 0.15 M NaCl/0.015 M sodium citrate; and TAT, tyrosine aminotransferase.

(DEX)-charged cytosol binding to isolated nuclei were determined.

Since the initial *in vivo* studies demonstrating inhibition of TAT enzyme activity by chemical carcinogens in rats [9], the inhibition of the steroid-inducible TAT gene product has been demonstrated by several groups using various carcinogens in hepatoma and primary liver culture systems [13–16]. Our laboratory has demonstrated conclusively that the carcinogen-mediated inhibition of TAT enzyme activity is the result of a decreased accumulation of TAT specific RNAs. Treatment of Reuber rat hepatoma cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) caused a 78% decrease in DEX-induced TAT enzyme activity, which was accompanied by a 75% inhibition of total DEX-inducible TAT-specific RNA [15]. This inhibition is limited to the specialized liver function TAT gene, as a subsequent study has shown that MNNG treatment does not alter the RNA levels of the constitutively expressed α-tubulin gene transcripts nor does it affect the DEX- and Cd-mediated induction of metallothionein RNA [17]. In addition, in this same study no effect of MNNG on the methylation pattern of the TAT gene was observed in studies employing the isoschizomeric restriction enzymes *Msp*I/*Hpa*II.

This laboratory has continued to study the possible mechanisms of carcinogen action on hormone-inducible gene expression with the intent to further delineate the molecular processes whereby oncogenic agents can alter the cell's normal program of gene expression during conversion to a neoplastic phenotype. However, the results obtained on RNA expression levels in cell culture have not been confirmed *in vivo*. In addition, the rat hepatoma models employed previously limit the choice of steroid-sensitive genes that can be examined, since not all steroid-inducible genes are expressed in these cultured cell lines. In this report, we have extended our earlier studies [15, 17] to the whole rat, and demonstrate that, as seen in the hepatoma culture system, treatment of rats with methylating carcinogens results in an inhibition in the accumulation of specific RNA species associated with steroid-inducible, specialized, liver function genes.

MATERIALS AND METHODS

Chemicals. Olive oil, DMN, bovine serum albumin (fraction V), Ficoll, and polyvinylpyrrolidone were purchased from the Sigma Chemical Co., St. Louis, MO; guanidine isothiocyanate was obtained from Bethesda Research Laboratories, Gaithersburg, MD; formamide was purchased from the Fluka Chemical Corp., Hauppauge, NY; glyoxal was obtained from the Fisher Scientific Co., Springfield, NJ; dextran sulfate was purchased from 5 Prime→3 Prime, Inc., Paoli, PA; and [α-^{32}P]dCTP (~3000 Ci/mmol) was obtained from the Amersham Corp., Arlington Heights, IL.

Animals and treatment protocols. Six-week-old CR(RAR) Sprague–Dawley rats were obtained from the Animal Production Area of the FCRDC. The rats were housed in a pathogen-free environment in plastic cages with hardwood shavings as bedding and were allowed free access to food (NIH-31 Open Formula Autoclavable Diet) and tap water. A 12-hr fluorescent light/dark cycle was maintained. Rats were pretreated by a single i.p. injection of either 2.25 mL/kg of phosphate-buffered saline (PBS) vehicle or 2.25 mL/kg of a 10 mg/mL DMN solution to yield a final carcinogen concentration of 22.5 mg/kg. Two hours after the initial pretreatment, the rats were then injected with either 3 mL/kg of a 50% ethanol vehicle or 3 mL/kg of a 0.45 mg/mL DEX solution to yield a final concentration of 1.35 mg/kg. Rats used for the receptor binding assays received only the initial 2-hr pretreatment of either PBS or DMN. Rats were euthanized by CO_2 asphyxiation during the late afternoon and early evening.

TAT enzyme and glucocorticoid receptor binding assays. TAT activity was determined by the assay described by Diamondstone [18]. The reactions were carried out in duplicate under conditions that were linear with respect to time and protein concentration, and saturating with respect to substrate concentration. Glucocorticoid receptor levels were determined by a filter binding assay as described previously [19]. Protein content was measured by the method of Bradford [20].

Isolation and characterization of total cellular RNA. RNA was purified as described by MacDonald *et al.* [21]. Livers were removed and placed immediately in a cold 4 M guanidine isothiocyanate/0.1 M Tris–HCl (pH 7.5)/1% 2-mercaptoethanol solution. The tissues were homogenized by a 60-sec burst with a Polytron homogenizer and then passed through an 18 gauge syringe needle six times to shear DNA and decrease the viscosity of the solution. Sodium sarkosyl (*N*-lauroylsarcosine, sodium salt) was added to a final concentration of 0.5% and the homogenates were layered over a 6.1 M CsCl/25 mM NaAc (pH 5.2)/10 mM EDTA solution. RNA was collected by centrifugation for approximately 20 hr at 20° in a Beckman SW41 rotor at 110,000 *g*. The RNA pellets were redissolved in 0.5 mL of autoclaved, glass-distilled, diethylpyrocarbonate-treated water and precipitated by centrifugation from a 70% ethanol/0.3 M NaAc (pH 5.2) solution. The pellets were rinsed with 70% ethanol and dried in a Speed-Vac. RNA was then dissolved in 10 mM sodium phosphate buffer (pH 7.0), denatured by treatment with glyoxal, and either blotted directly onto a Biodyne A nylon membrane filter (Pall Ultrafine Filtration Corp., Glen Cove, NY) using a Schleicher & Schuell Minifold II apparatus or first fractionated by electrophoresis in a 1% agarose gel with constant recirculation of the 10 mM sodium phosphate (pH 7.0) buffer [22].

The blots were baked, prehybridized, and hybridized as described [22], except that the prehybridization and hybridization incubations were performed at 50°. Plasmid pUTAT, a 940 bp genomic clone of the rat TAT gene [23], was provided by Dr. Günther Schütz; pGSRK-1, a cDNA clone of the rat glutamine synthetase (GS) gene, was provided by Dr. Richard Miller [24]; and LK345, a genomic clone of the human β-actin gene, was obtained from Dr. Larry Kedes [25]. The plasmids were labeled by the random primer labeling technique [26]. Following hybridization, the blots were washed three times

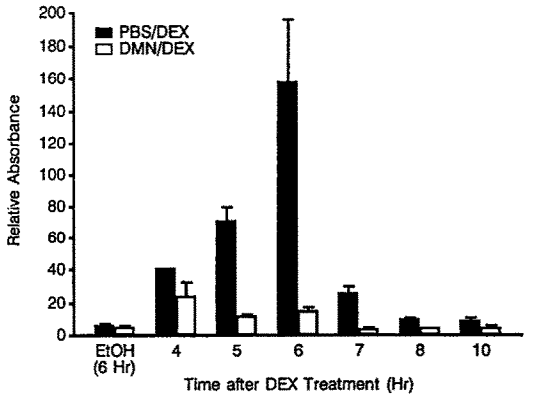

Fig. 1. DMN-mediated inhibition of DEX-inducible TAT RNA accumulation. Sprague–Dawley rats were pretreated with a single i.p. injection of either 2.25 mL/kg of PBS or 22.5 mg/kg of DMN, followed 2 hr later by a single i.p. injection of 1.35 mg/kg of DEX. At various times after the DEX injection, the rats were euthanized by CO_2 asphyxiation and RNA was purified and blotted to nylon membranes. The membranes were probed with the pUTAT plasmid, and the autoradiographs analyzed by laser densitometry. Values are reported as relative absorbance and represent the means ± SEM from three rats. Values lacking error bars are the averages from two rats. Expression of TAT RNA was corrected for β-actin RNA levels. Groups marked EtOH were treated with 3 mL/kg of an ethanol vehicle instead of DEX for 6 hr.

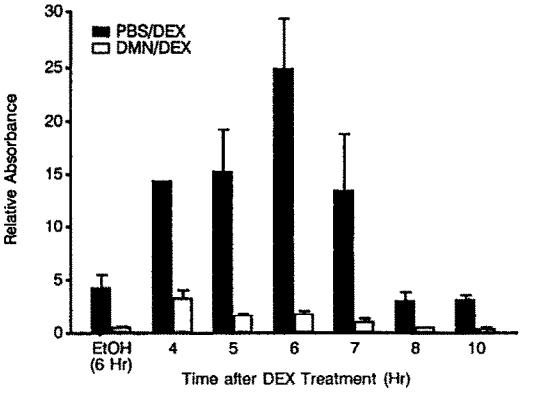

Fig. 2. DMN-mediated inhibition of DEX-inducible GS RNA accumulation. Sprague–Dawley rats were pretreated with a single i.p. injection of either 2.25 mL/kg of PBS or 22.5 mg/kg of DMN, followed 2 hr later by a single i.p. injection of 1.35 mg/kg of DEX. At various times after the DEX injection, the rats were euthanized by CO_2 asphyxiation and RNA was purified and blotted to nylon membranes. The membranes were probed with the pGSRK-1 plasmid, and the autoradiographs analyzed by laser densitometry. Values are reported as relative absorbance and represent the means ± SEM from three rats. Values lacking error bars are the averages from two rats. Expression of GS RNA was corrected for β-actin RNA levels. Groups marked EtOH were treated with 3 mL/kg of ethanol vehicle instead of DEX for 6 hr.

with 2 × SSC/0.1% SDS at 50° for 5–10 min followed by three washes with 0.1 × SSC/0.1% SDS at 50–52° for 15 min. The blots were wrapped in Saran-Wrap and autoradiographed in the presence of an intensifying screen (Cronex Lightning Plus, Dupont, Wilmington, DE) with preflashed Kodak X-Omat XAR-5 film at −80°.

RESULTS AND DISCUSSION

For our studies on the effects of DMN on steroid-inducible gene expression, we initially treated the rats with a single i.p. injection of either vehicle or a 22.5 mg/kg dose of DMN, followed 2 hr later by treatment with a single i.p. injection of either the 50% ethanol vehicle or a 1.35 mg/kg dose of DEX. The timing of the injections and doses used in this study have been shown to coincide with the maximal induction of TAT enzyme activity by DEX and the maximal inhibition by DMN and AFB of the DEX-induced increase in TAT enzyme activity [9, 10]. Preliminary studies conducted in this laboratory confirmed these earlier studies (data not shown). Rats pretreated for 2–3 hr with either vehicle or DMN followed by treatment with either 50% ethanol or DEX for 6 hr had the following enzyme activities, expressed as the means ± SEM for TAT activity in nmol of *p*-hydroxyphenylpyruvic acid formed/min/mg protein (results from three experiments): PBS/ethanol: 20 ± 7; PBS/DEX: 142 ± 24; and DMN/DEX: 52 ± 13. This represented a 12 ± 6-fold induction of TAT activity in PBS/DEX-treated rats compared to PBS/ethanol-treated controls. DMN pretreatment resulted in a 64 ± 4% decrease in DEX-induced TAT activity.

To analyze the effects of DMN on the accumulation of steroid-inducible RNAs, rats were pretreated for 2 hr with either PBS vehicle or DMN, followed by treatment with either the 50% ethanol vehicle or 1.35 mg/kg DEX. Rats were euthanized at various times after DEX treatment and the RNAs isolated and analyzed as described in Materials and Methods. Maximal levels of TAT (26-fold) and glutamine synthetase (GS) (6-fold) RNAs were observed 6 hr after DEX administration. Cotreatment of rats with DMN/DEX resulted in marked decreases in the levels of both RNA species relative to rats treated with PBS/DEX (Figs. 1 and 2). DMN caused maximal relative inhibition of both TAT and GS RNA accumulation at 6–7 hr after DEX administration, when the levels of RNAs were decreased to 9–13 and 7–8% of PBS/DEX-treated rats, respectively. Thus, as has been demonstrated with the inhibition of TAT enzyme activity by DMN ([10]; this study), maximal inhibition by DMN occurred at the time of the peak inductive response to DEX stimulation. By 8 hr after DEX administration, pretreatment with DMN caused much less of an inhibitory effect, with DMN/DEX-treated TAT RNA levels recovering to 45% of PBS/DEX levels, while DMN/DEX GS RNA levels were still only 18% of PBS/DEX levels. As discussed below, ethanol appeared to act synergistically with DMN to inhibit basal GS RNA expression (Fig. 2). This may account, in part, for the marked inhibition of GS RNAs in DMN/DEX-treated rats.

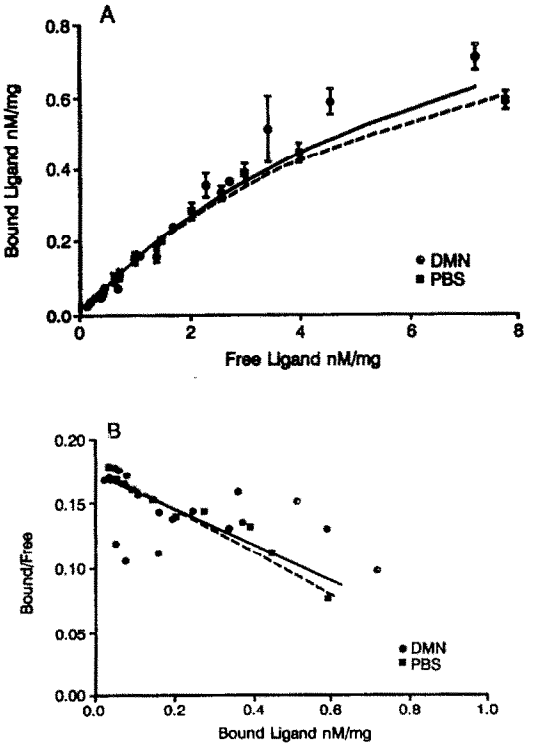

Fig. 3. Effect of DMN on binding of DEX to the glucocorticoid receptor. Sprague–Dawley rats were treated with a single i.p. injection of either 2.25 mL/kg of PBS or 22.5 mg/kg of DMN. Two hours later the rats were euthanized by CO_2 asphyxiation. Binding of [^{3}H]DEX to rat cytosols was determined by a filter binding assay. Values are the means ± SEM from three experiments with cytosols isolated from individual animals for each experiment. The results for each individual experiment were from duplicate determinations. (A) Binding of [^{3}H]DEX to rat cytosols from vehicle or DMN-treated rats; (B) Scatchard analysis of the data from Fig. 3A.

Table 1. Densitometric analysis of RNAs from vehicle-treated rats

Treatment	Relative absorbance units	
	TAT RNA	GS RNA
None	2.72 ± 0.66	1.14 ± 0.13
PBS	9.24 ± 1.90	3.60 ± 0.90
PBS/ethanol	6.19 ± 1.07	4.23 ± 1.14
DMN	11.99 ± 2.60	2.63 ± 0.83
DMN/ethanol	4.76 ± 0.70	0.49 ± 0.06

Sprague–Dawley rats were either left untreated, treated with a single i.p. injection of 2.25 mL/kg of PBS or 22.5 mg/kg of DMN for 2 hr, or treated with PBS or DMN followed 2 hr later by treatment with a single i.p. injection of 3 mL/kg of a 50% ethanol solution for 6 hr. RNAs were isolated and blotted directly to nylon membranes and probed with the pUTAT, pGSRK-1, and LK345 plasmids. The data obtained with the pUTAT and pGSRK-1 plasmids were corrected for β-actin expression. All values are expressed as relative absorbance units following densitometric scans of the slot blots, and are the means ± SEM from at least three individual rats.

The inhibition of DEX-induced TAT and GS RNAs by DMN did not appear to be mediated through alterations in glucocorticoid receptor (GCR)–ligand complex formation (Fig. 3). Scatchard analysis of receptors from the cytosols of rats treated *in vivo* with either PBS or DMN for 2 hr showed that DMN had little effect on either the K_d or B_{max} of the receptors. GCRs from PBS treated rats had a K_d of 0.162 ± 0.013 nM/mg and a B_{max} of 1.102 ± 0.067 nM/mg, while DMN-treated rat cytosols had values of 0.138 ± 0.012 and 1.261 ± 0.103 nM/mg, respectively (Fig. 3B). These results agree well with our previous studies in rat hepatoma cells, which showed that treatment with MNNG inhibited induction of TAT RNA but had no effect on the induction of metallothionein RNA, implying the presence of fully functional receptors [17]. In addition, Kensler *et al.* [11] obtained similar results with *in vivo* rat models using AFB as the carcinogen. Horikoshi *et al.* [16] have reported that treatment of rat hepatoma cells with AFB and sterigmatocystin may alter GCR structure and function. However, these authors treated cytosolic fractions of the cells directly with carcinogens, which may not accurately reflect processes that occur in whole cells or *in vivo*.

In comparing the results obtained with the TAT (Fig. 1) and GS (Fig. 2) genes, it appeared that the GS gene was more sensitive to DMN. We therefore examined the effects of the vehicles on the basal levels of expression of these two genes. Compared to untreated rats, treatment with either PBS alone or PBS followed 2 hr later by ethanol (duration of ethanol treatment was 6 hr) resulted in a 2.2- to 3.7-fold increase in the levels of both RNA species, with little difference between animals treated with PBS alone or PBS/ethanol, although TAT RNA levels in the ethanol-treated animals were 33% lower than rats receiving only the PBS (Table 1). These increased levels of inducible RNAs may result from the release of endogenous glucocorticoids in response to the stress of the i.p. injections, as these rats contain intact adrenal glands. Treatment with DMN alone had little effect on either TAT or GS RNA compared to PBS-treated rats. However, treatment with the DMN/ethanol combination produced an 88% decrease in the levels of GS RNA compared to PBS/ethanol-treated animals. While only a 23% reduction in the equivalent TAT RNA groups was observed, DMN/ethanol-treated rats had 60% lower basal TAT RNA levels than rats treated with DMN alone. These results suggest that ethanol may act to enhance the inhibitory effects of DMN on both basal and inducible RNA expression. The mechanism of this interaction is currently unknown, but probably accounts, at least in part, for the greater inhibition of steroid-inducible GS RNA accumulation compared to inducible TAT RNA accumulation.

These experiments show that the results obtained in the rat hepatoma cell lines accurately reflect processes that occur in the whole animal, suggesting that future mechanistic studies can be carried out in

the hepatoma cell clones. Results obtained in previous studies have demonstrated that the inhibition of steroid-inducible gene expression in hepatoma cell culture systems appears to be a specific effect limited to specialized function genes, as expression of the ubiquitous steroid and metal sensitive metallothionein gene was not affected by carcinogen treatment [17]. Other laboratories have also demonstrated rather specific effects of carcinogens on specialized hormone sensitive genes as compared to overall effects on gene expression [7, 13, 27]. In addition, chemical carcinogens had little or no effect on the basal levels of gene expression compared to induced levels of expression in several studies by independent laboratories [9, 10, 14, 15]. However, further experimentation needs to be done in the *in vivo* rat model with RNAs expressed by other ubiquitous and specialized function genes, particularly those with short half-lives, to further determine the specificity of this effect.

The ability of chemical carcinogens to down-regulate the expression of differentiation-associated specialized genes may be mediated by the same mechanisms responsible for the increased expression of oncogenes. Chemical carcinogens have been shown to cause mutations in DNA sequences, leading to alterations in oncogene expression and/or function. Hormone-responsive genes may be particularly good targets for carcinogen action since transcriptionally active genes may be preferential targets for these chemicals [28–33]. Courey *et al.* [34] have shown that SV-40 enhancer-dependent transcription is inhibited when a psoralen-modified SV-40/human globin gene recombinant construct is transfected into HeLa cells, thus demonstrating that placement of an adduct anywhere between the enhancer and promoter regions of the gene can cause marked inhibition of enhancer-dependent gene transcription. This would present a rather large target for the chemical carcinogen and could partially explain the preferential targeting of these types of gene systems.

In addition to direct damage to genomic DNA, carcinogens may also alter gene transcription by interfering with the binding of *trans*-acting regulatory proteins to DNA. Several laboratories have identified liver-specific regulatory proteins that control the transcriptional activity of specialized liver function genes [35–37]. If carcinogenic agents can inhibit the proper functioning of these proteins, it is conceivable that the transcriptional activity of a whole array of liver specific genes could be affected.

Several studies have established an inverse correlation between protooncogene expression, which is often elevated in tumor tissue, and expression of glucocorticoid-sensitive genes. Vacca *et al.* [38] have shown that overexpression of Ha-*ras* inhibits transcription from the mouse mammary tumor virus long terminal repeat. Other studies have demonstrated that c-*jun* and c-*fos*, members of the AP-1 family of transcriptional activators, can interfere with transcriptional enhancement by preventing binding of the ligand-activated GCR complex to glucocorticoid response elements through direct competition at DNA regulatory sites [39–41]. Gurney *et al.* [42] have shown that increased expression of Fos/Jun heterodimers blocks inducible transcription from the phospho*enol*pyruvate carboxykinase gene. Overexpression of several oncogenic proteins, including Src, Ras, Mos, and Fos has also been shown to inhibit differentiation of mammary epithelial cells [43]. Increased levels of AP-1 activity have been implicated in this effect as well.

In addition to their effects on gene transcription, other studies from this laboratory have shown that chemical carcinogens can mediate their inhibitory effects on gene expression at the post-transcriptional level in some cell types [44]. Thus, the down-regulation of hormone-responsive, specialized, liver function genes may be part of a pleiotropic program initiated during tumor progression.

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