MOLECULAR AND CELLULAR BIOLOGY, Aug. 1990, p. 4389–4395
0270-7306/90/084389-07$02.00/0

Vol. 10, No. 8

The Permissive Role of Glucocorticoids on Interleukin-1 Stimulation of Angiotensinogen Gene Transcription Is Mediated by an Interaction between Inducible Enhancers

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Received 22 March 1990/Accepted 9 May 1990

The acute-phase activation of the rat angiotensinogen (rAT) gene in liver cells is a transcriptional event mediated through an interleukin-1-inducible, NFκB-binding, *cis*-acting element (the acute-phase response element [APRE]). Using a cell culture model for the acute-phase response, we showed that the increase in angiotensinogen mRNA in H35 rat hepatoma cells requires costimulation with glucocorticoids and cytokines. Stably transfected rAT promoter-luciferase reporter genes were also activated by cytokines only in the presence of glucocorticoids. This permissive role of glucocorticoids is dependent on the expression of functional glucocorticoid receptors, because in HepG2 cells naturally deficient in such receptors, rAT gene-luciferase reporter constructs responded to interleukin-1 only when cotransfected with an expression vector for the glucocorticoid receptor. Point mutations in the two rAT gene glucocorticoid response elements located adjacent to the APRE led to loss of interleukin-1 inducibility. Induction of luciferase activity in transfected cells occurred even in the presence of cycloheximide, demonstrating that this synergistic response did not depend on new protein synthesis. Thus, a direct interaction between the interleukin-1-inducible NFκB-binding APRE and glucocorticoid response elements, located in *cis*, underlies the acute-phase activation of the rAT gene.

The hepatic acute-phase response induces changes in liver secretion rates for proteins involved in macrophage opsonization and wound repair. Consistent with the limited capacity for storage of preformed protein in the liver, the acute-phase response has been shown, by in vitro transcription run-on assays, to reflect a change in the transcription rates of the genes coding for the various acute-phase response proteins (7). This transcriptional event is mediated by an inflammation-induced increase in circulating levels of cytokines and glucocorticoid hormones (for reviews, see references 15 and 23). Studies with live animals and experiments with primary cultures of hepatocytes, as well as with various transformed hepatic cell lines, have established the existence of overlapping sets of acute-phase response genes regulated by interleukin-1 (IL-1) and tumor necrosis factor, by IL-6 and other hepatocyte-stimulating factors, by glucocorticoids, or by a combination of the above (for a review, see reference 2). A subset of these genes is under dual regulation by both cytokines and glucocorticoids. The latter category is exemplified by the IL-1-responsive rat α_1 acid-glycoprotein (4), by the IL-6-responsive rat α_2 macroglobulin (35), and by the alpha, beta, and gamma genes of rat fibrinogen (26). This subset of genes provides an interesting example of dual regulation of gene transcription by two distinct hormonal systems (cytokines and adrenal glucocorticoids) that are in turn coordinately responding to a single physiological event in the life of the organism.

The rat angiotensinogen (rAT) gene that codes for the only known precursor of the potent vasoactive peptide, angiotensin II, is a well-established hepatic acute-phase response gene (8, 19, 30) and is also regulated by glucocorticoids at the transcriptional level (9, 12). We have previously identified a cytokine-responsive acute-phase response element (APRE) in the 5′-flanking region of the rAT gene. This *cis*-acting DNA element, centered on an 18-base-pair palindrome (nucleotides [nt] −552 to −537 from the transcription start site [numbering as in reference 25]), binds a 50-kilodalton NFκB-like IL-1-inducible protein, as well as a 32-kilodalton protein constitutively present in the nucleus (30). The rAT gene APRE is flanked on both sides by classic glucocorticoid response elements (GREs). These two GREs, centered on nt −557 and −475, respectively, impart glucocorticoid inducibility on the rAT gene through a synergistic interaction with each other (A. R. Brasier et al., submitted for publication). In the study presented here, we identified a functional dependence of rAT gene transcription induction by IL-1 on concurrent stimulation by dexamethasone and demonstrated that this phenomenon is due to an interaction, in *cis*, between the glucocorticoid-inducible GREs and the cytokine-inducible APRE.

Glucocorticoids are necessary for cytokine induction of rAT mRNA in hepatoma cells. We have shown previously that stimulation of Reuber H35 cells (a well-differentiated rat hepatoma cell line) with conditioned medium from lipopolysaccharide-treated mouse monocytic cells, which are known to be an abundant source of IL-1 and tumor necrosis factor (5), leads to an increase in rAT mRNA levels (30). Thus, the Reuber H35 cell line is a useful cell culture model for studying acute-phase activation of the rAT gene. Previous cytokine stimulation experiments had been performed in the presence of glucocorticoids in the culture medium, which served to increase the baseline rAT mRNA levels. In the present study, H35 cells grown to confluence in Dulbecco modified Eagle medium supplemented with 10% charcoal-treated fetal calf serum (31) were stimulated for 4, 8, or 24 h with 0.4% (vol/vol) 10-fold diafiltration-concentrated conditioned medium from lipopolysaccharide-treated Raw 264.7 monocytic cells (RawCM, as described in reference 30) with or without 24 h of costimulation with 5×10^{-7} M dexamethasone. Northern (RNA) blot analysis of total cellular

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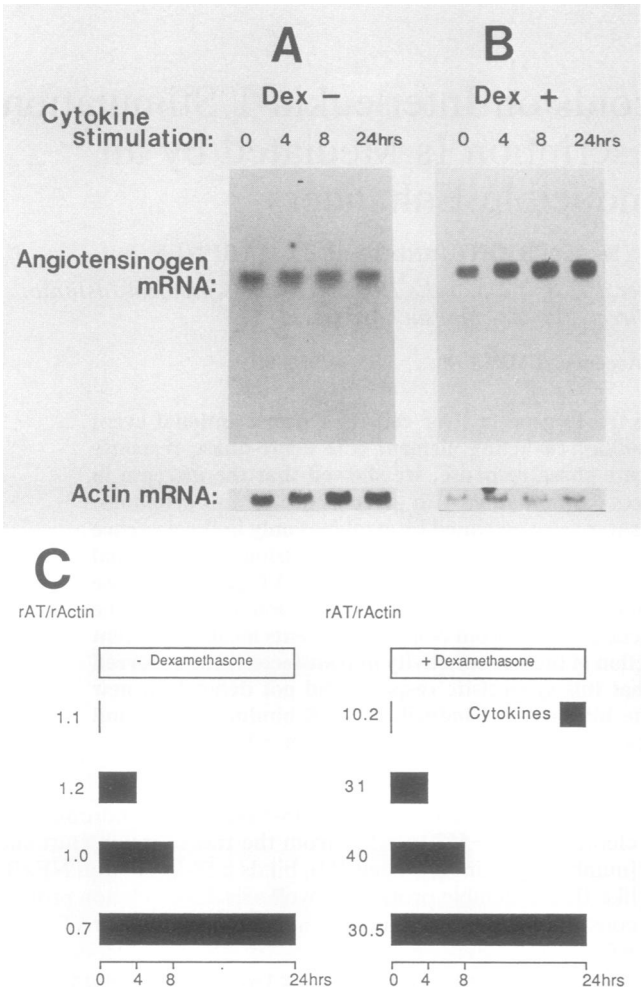

FIG. 1. Dependence of induction of rAT mRNA by monocyte-conditioned medium on costimulation with dexamethasone. (A and B) Top panel is a Northern blot of total cellular RNA from H35 cells hybridized with rAT gene cDNA. Bottom panel is the identical blot probed with oligonucleotides complementary to actin mRNA. Cells were stimulated for the indicated number of hours with 0.4% (vol/vol) RawCM prior to harvest (at time zero). Northern blots represent 20 μg of RNA from cells stimulated in the absence of dexamethasone (DEX −) (A) and 5 μg of RNA from cells stimulated in the presence of 5×10^{-7} M dexamethasone (DEX +) (B). (C) Densitometric quantitation of the ratios between the rAT and actin mRNA signals (panels A and B) following stimulation of H35 cells with conditioned medium containing cytokines for 4, 8, and 24 h. Stimulations were carried out in the absence or continuous presence of dexamethasone, as indicated.

RNA with the rAT gene cDNA and oligonucleotides complementary to rat actin mRNA as probes was performed (Fig. 1). In the absence of dexamethasone, no cytokine-induced increase in rAT mRNA was detectable (Fig. 1A). Stimulation with conditioned medium in the presence of dexamethasone led to the expected three- to fourfold cytokine induction of rAT mRNA, which appeared by 4 h, as determined by densitometric scanning of the autoradiograms (Fig. 1B and C). The permissive role of glucocorticoids in cytokine induction of the rAT gene was seen when the two agents were added to the cells simultaneously (see below). However, in order not to confound the interpretation of the results of the cytokine stimulation by the increase in rAT gene transcriptional activity that occurs within the first 12 h of treatment with dexamethasone alone (12), the cytokine induction was performed at the end of the 24-h period, after the addition of dexamethasone to the culture medium; in other words, it was performed at a time in which glucocorticoid activation of rAT gene transcription had already reached steady-state levels.

The magnitude and kinetics of cytokine induction were similar to those observed in vivo in livers of rats injected with bacterial lipopolysaccharide (8, 19, 30). The increase in rAT mRNA in response to 24 h of continuous stimulation with dexamethasone alone can be appreciated by comparing the ratios of the intensity of rAT to actin signals in the cells untreated with conditioned medium (Fig. 1A and B, lanes 0; both were exposed to give a comparable intensity to the pre-cytokine-stimulated rAT signal). The response to dexamethasone and cytokines was quantified by densitometric scanning of the autoradiogram (Fig. 1C). The observed 10-fold induction of rAT mRNA by dexamethasone alone is consistent with published results obtained with the same experimental system (12). Thus, although rAT gene expression is induced by dexamethasone in the absence of cytokines, the converse is not true. Glucocorticoids therefore exert a permissive role on the cytokine induction of the rAT gene.

A 688-base-pair fragment of the rAT gene 5′-flanking region exhibits glucocorticoid-dependent cytokine inducibility. The rAT gene APRE located between nt −552 and −537 (Fig. 2A) functions as an IL-1- and tumor necrosis factor-inducible enhancer in gene transfer experiments in hepatoma cell lines. Cytokine induction of reporter plasmids containing multiple copies of the APRE upstream of a minimal promoter, however, is not dependent on costimulation of the cells with dexamethasone (30). Therefore, we sought to determine whether the dexamethasone dependence of cytokine stimulation of the endogenous rAT gene was due to the particular arrangement of the APRE in the context of the rAT promoter. The cytokine responsiveness of H35 cells that were stably transformed with a luciferase reporter gene driven by an rAT gene fragment spanning nt −688 to +39 was tested in the absence and presence of dexamethasone. Following 4 h of treatment with RawCM, the reporter gene activity was increased only in cells costimulated with dexamethasone (Fig. 2B). The glucocorticoid dependence of cytokine stimulation was dose related and was similar in magnitude to that seen in the endogenous rAT gene. The same results were obtained in two other independently derived, stably transformed H35 clones (data not shown), demonstrating that the dexamethasone dependence of cytokine induction is unlikely to be a consequence of the particular integration site of the reporter gene. Reconstruction of the dexamethasone dependence of cytokine stimulation in a chimeric reporter construct was consistent with a transcriptional mechanism and prompted us to dissect further the rAT gene regulatory sequences involved in this synergistic response.

The permissive role of glucocorticoids requires a ligand-activated glucocorticoid receptor and a functional rAT GRE in *cis*. To study the role of the previously identified two rAT GREs and the APRE in the dexamethasone dependence of rAT gene cytokine inducibility, we turned to transient transfection assays of wild-type and mutant reporter constructs in a regulated hepatic cell line. Because of their poor transfectability, the H35 cells proved difficult to reliably assay in transient transfections, so the more readily transfectable, angiotensinogen-expressing human hepatoblastoma cell line HepG2 (11) was used. Consistent with our observations that

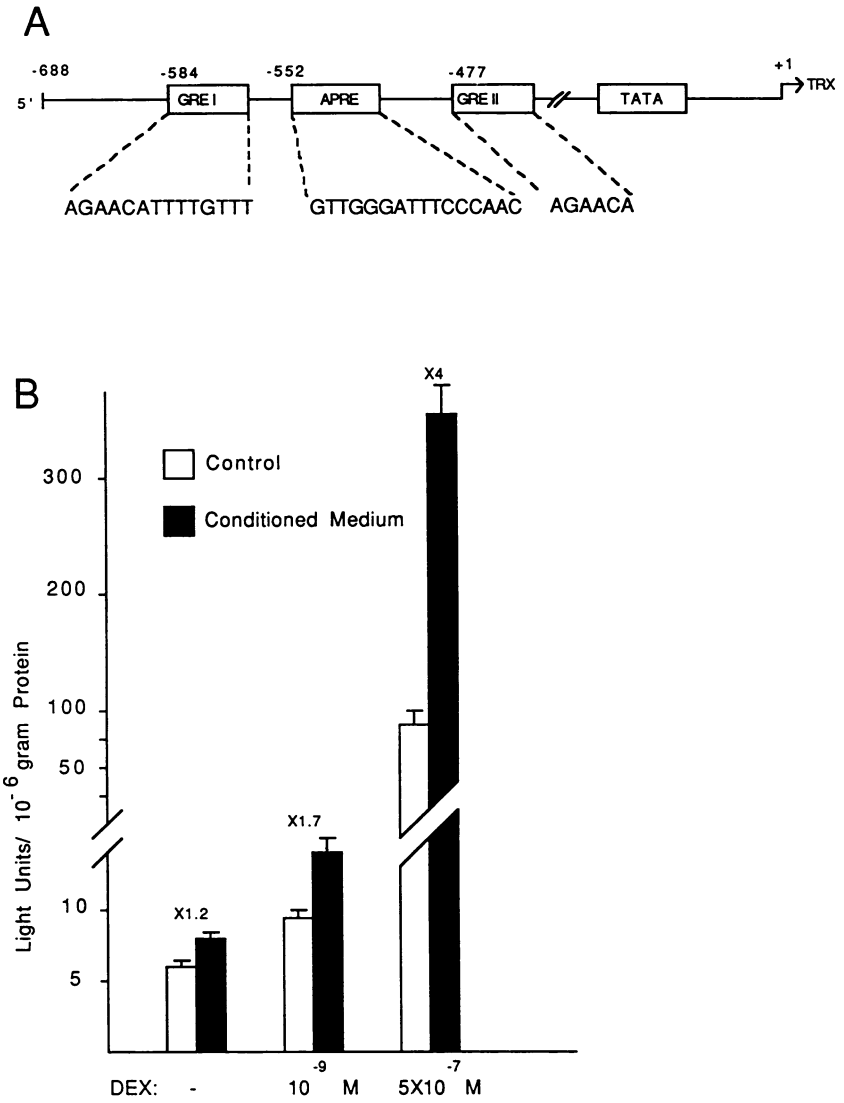

FIG. 2. Glucocorticoid-dependent activation of rAT gene promoter by cytokines in gene transfer experiment. (A) Relative positions and sequences of the rAT APRE and two GREs. (B) Luciferase activity of H35 cells stably transformed with p688RLG and stimulated with RawCM for 4 h in the presence of different concentrations of dexamethasone. Luciferase activity was normalized to the protein content of the cell lysate. Numbers above columns represent fold stimulation by 0.4% (vol/vol) RawCM. Data and error bars show means and ranges of experiments performed in duplicate and repeated at least three times.

HepG2 cells are deficient in functional glucocorticoid receptors (Brasier et al., submitted), we noted that the endogenous angiotensinogen gene of these cells was not activated by cytokines or glucocorticoids (data not shown). This deficiency, however, allowed us to assay for the role of the glucocorticoid receptor by selective cotransfection of an expression plasmid encoding the human glucocorticoid receptor.

Transient transfections into HepG2 cells and luciferase enzymatic activity assays were carried out as reported previously (10). Reporter plasmid DNA (10 μg) along with expression vector (2 μg) for the human glucocorticoid receptor (RShGR; a gift of Ron Evans [17]), internal control plasmid (2 μg) expressing a placental alkaline phosphatase reporter gene from the simian virus 40 early-early promoter (pSV2PAP; a gift of Tom Kadesch [18]), and inert vector plasmid DNA (pGEM; Promega Biotec, Madison, Wis.) to a total of 20 μg was used to transfect batches of four 60-mm-diameter tissue culture plates by the calcium phosphate coprecipitation technique. After 24 h, the medium was replaced with fresh medium, and 20 h later, the cells were stimulated with human recombinant IL-1α (a gift of Steven Gillis, Immunex Corp., Seattle, Wash.) with or without 5×10^{-7} M dexamethasone for 4 h prior to harvest. Reporter plasmids used to study the interaction between the APRE and the GREs were constructed by cloning a genomic fragment of the rAT gene from nt −615 to −442 upstream of a minimal rAT promoter capable of faithfully initiating transcription (p59RLG [11]). The resulting plasmid carrying the rAT inducible enhancer unit (IEU) was termed IEUp59RLG. Direct-site mutagenesis of the inducible enhancer unit was achieved by cloning the aforementioned genomic fragment into an M13 vector and creating specific base pair substitutions by oligonucleotide-directed mutagenesis (22). In the APRE, nucleotides critical for the binding of both previously identified nuclear proteins (30) were substi-

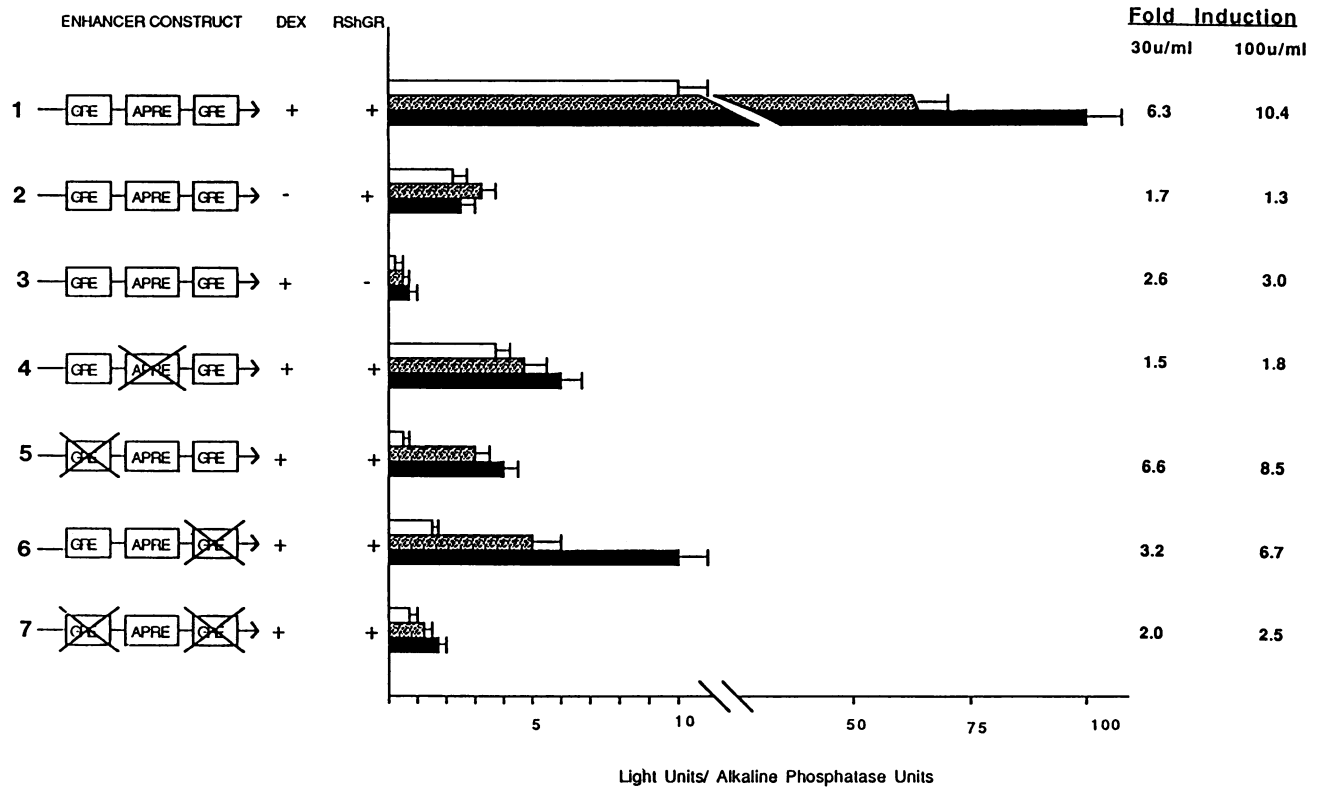

FIG. 3. Dependence of rAT induction by IL-1 on an active GRE- and ligand-activated glucocorticoid receptor. HepG2 cells transiently transfected with the indicated enhancer-containing luciferase-reporter constructs, with or without an expression vector for the human glucocorticoid receptor (RShGR), were stimulated for 4 h with 30 U (▨) or 100 U (■) of IL-1 per ml (□, no IL-1) in the presence or absence of 5×10^{-7} M dexamethasone (DEX). IL-1 activation of luciferase reporter activity normalized to activity of cotransfected reference plasmid, pSV2PAP, is expressed as arbitrary light units, normalized to placental alkaline phosphatase activity. Error bars represent the range of values of experiments performed in duplicate and repeated at least twice.

tuted, converting the wild-type sequence GTTGGGATTTC CCAAC to the mutant sequence TGTTGGATTTCCGATA. The mutations of the upstream and downstream GREs were two point mutations in the conserved hexamer which abolish binding of the glucocorticoid receptor, converting the wild-type sequence AGAACA to ATAAAA.

In the presence of dexamethasone and when cotransfected with RShGR, the wild-type reporter construct, IEUp59RLG, responded with a dose-dependent increase in luciferase activity to stimulation with IL-1 (Fig. 3, construct 1). The response was rapid, occurring within 4 h, with a 6-fold increase in response to 30 U/ml and a 10-fold increase to 100 U/ml, and was similar to the acute-phase activation of the rAT gene seen in rats and in H35 cells. Thus, in terms of mimicking the acute-phase regulation of the rAT gene, an inducible enhancer unit composed of the APRE and the GREs is sufficient. Activation of this inducible construct was shown to be dependent on dexamethasone, because in its absence, only a minimal (1.3-fold) induction by IL-1 was noted (Fig. 3, construct 2).

The permissive role of dexamethasone was shown to be effected through its interaction with its specific receptor, because omission of the glucocorticoid receptor expression plasmid led to a marked attenuation of the permissive effect of dexamethasone on IL-1 induction of reporter activity (Fig. 3, construct 3). Residual IL-1 stimulation, in the absence of cotransfected RShGR, most likely represents the effect of minimal levels of endogenous receptor present in the cells, because it too was dependent on glucocorticoid stimulation (data not shown).

The role of dexamethasone in IL-1 induction of the rAT gene did not appear to depend simply on the ability of glucocorticoids to increase basal transcriptional activity (prestimulation by cytokines) of the reporter constructs. This was demonstrated by the preserved IL-1 inducibility of reporter constructs lacking either one of the two rAT gene GREs (Fig. 3, mutant constructs 5 and 6). These single-GRE mutant constructs are only minimally responsive to stimulation with glucocorticoids (even though either GRE is capable, when dimerized and placed sufficiently close to a minimal promoter, of dexamethasone-dependent activation of transcription [Brasier et al., submitted]). Consistently, they had a pre-cytokine-stimulation activity equal to or lower than that of the wild-type construct, even in the absence of dexamethasone (Fig. 3, construct 2, compared with constructs 5 and 6). However, both single-GRE mutant constructs were not significantly different from the wild-type constructs in terms of their fold inducibility by IL-1 (Fig. 3, constructs 1, 5 and 6), a circumstance that is dependent on the presence of dexamethasone (data not shown).

Conclusive evidence that glucocorticoids exert their permissive role predominantly through a receptor-mediated interaction with a GRE in *cis* to the APRE was provided by the construct bearing a mutation of both functional GREs (Fig. 3, construct 7). This mutation led to a loss of most of the IL-1 responsiveness. It follows, therefore, from the

conserved IL-1 inducibility of constructs bearing a mutation in one of the two GREs, that a single GRE, in *cis*, is sufficient for the permissive effect of glucocorticoids. Residual IL-1 inducibility of the double-mutant construct (two- to threefold inducibility) is consistent with the existence of an ancillary facilitatory role of glucocorticoids on IL-1 induction that is not dependent on the presence of GREs in *cis* to the APRE or, alternatively, on the presence of cryptic GREs in the rAT promoter.

The synergistic interaction between glucocorticioids and cytokines depends on activation mediated through preformed proteins. Ligand-dependent activation of GREs (29) and cytokine activation of the rAT gene APRE (30) both proceed in the absence of new protein synthesis. We therefore determined whether the permissive role of glucocorticoids in cytokine induction of the rAT gene regulatory sequences would be affected in the presence of concentrations of cycloheximide known to inhibit protein synthesis. Independence from a requirement for protein synthesis would be consistent with a direct interaction between the two inducible enhancers, whereas dependence on protein synthesis would indicate an indirect mechanism of action.

HepG2 cells transfected with IEUp59RLG and pSV2PAP were stimulated with IL-1 and dexamethasone for 4 h in the continued presence of cycloheximide (10 μg/ml) that had been added to the medium 30 min earlier. Primer extension analysis of poly(A)$^+$ RNA using primers complementary to the luciferase-derived and pSV2PAP-derived transcripts was performed as described previously (30). We noted a fourfold increase in the abundance of correctly initiated reporter gene transcripts in response to 100 U of IL-1 per ml in the presence of 5×10^{-7} M dexamethasone (Fig. 4, compare lanes 4 and 5), confirming that the dexamethasone-dependent IL-1 induction of rAT gene transcription does not depend on new protein synthesis. In the absence of dexamethasone, the abundance of transcripts was very low (lane 3), confirming that induction of rAT transcription by dexamethasone alone also proceeds in the absence of new protein synthesis. Primer extension analysis with a primer directed to the transcript expressed from the cotransfected pSV2PAP internal recovery marker allowed us to control for transfection efficiency and RNA recovery, demonstrating that the measured increase in reporter gene activity is due to a true increase in abundance of its mRNA.

In the physiological context of tissue inflammation, circulating levels of cytokines and glucocorticoids increase in a coordinate and interrelated manner. IL-1 induces pituitary secretion of adrenocorticotrophic hormone, leading to an increase in circulating levels of glucocorticoids (6). Glucocorticoids in turn modulate secretion of IL-1 from inflammatory cells (33). The interrelatedness of these two effectors of the acute-phase response was shown in this study to extend to their interaction in activating rAT gene transcription through a functional inducible enhancer unit which contains two interdependent GREs and an APRE.

The rAT gene inducible enhancer unit appears to be composed of at least three interdependent functional inducible regulatory elements: two GREs and a cytokine-responsive APRE. These enhansons, which are insignificantly weak individually, provide for a substantial inducible activation of a downstream promoter when combined in the context of the inducible enhancer unit. Such modular organization of an enhancer unit has been best described in the case of the simian virus 40 early gene. Furthermore, an NFκB-binding site, similar to the rAT gene APRE and devoid in and of itself of independent enhancer activity, has been shown to be an important functional part of the simian virus 40 enhancer (20).

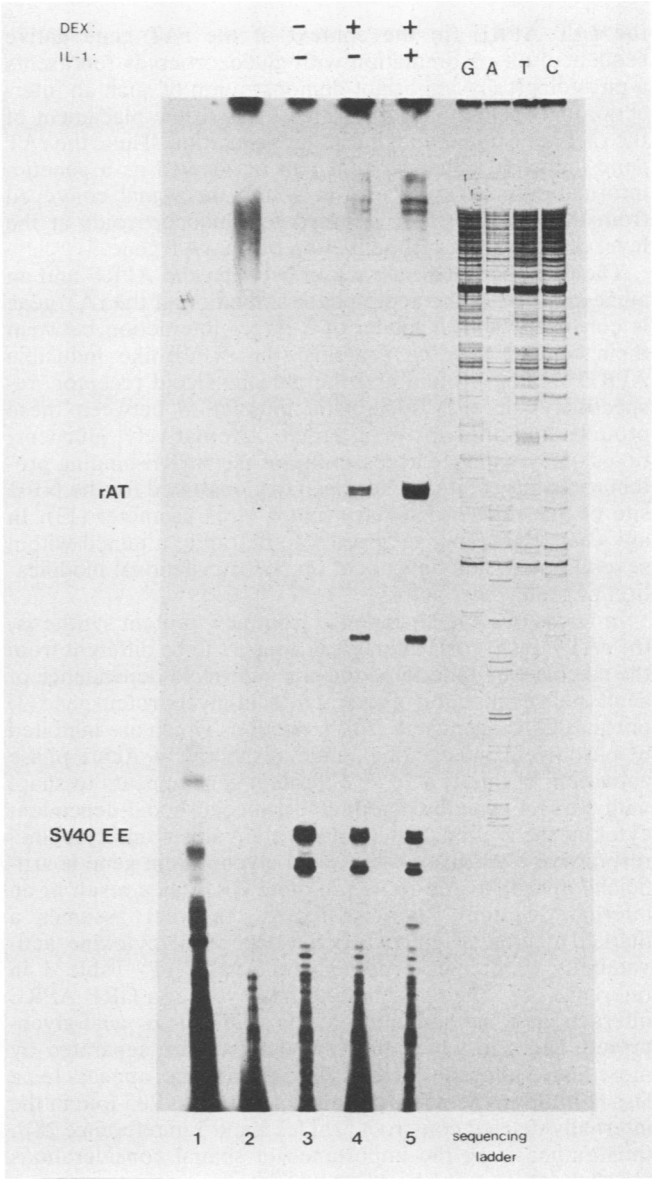

FIG. 4. Synergistic coinduction of rAT transcription by glucocorticoids and IL-1 not dependent on new protein synthesis. Shown is a primer extension analysis of poly(A)$^+$ RNA from HepG2 cells transfected with the luciferase reporter construct IEUp59RLG and an expression vector for the glucocorticoid receptor (RShGR). Transfected cells were stimulated for 4 h with either 100 U of IL-1 per ml and 5×10^{-7} M dexamethasone (lane 5) or dexamethasone alone (lane 4) or were not stimulated (lane 3), but all were treated with 10 μg of cycloheximide per ml. The position of the correctly initiated rAT-luciferase fusion transcript (rAT) and the transcript initiated from the cotransfected pSV2PAP internal control plasmid (SV40 EE) are indicated to the left. Primer extension of yeast tRNA (lane 1) and RNA from nontransfected HepG2 cells (lane 2) are included as further controls. A dideoxynucleotide sequencing reaction of the IEUp59RLG reporter plasmid using the same primer as that used for the primer extension assay is included on the right.

GREs have been shown in the past to interact promiscuously with other enhansons present in *cis*, exposing their latent activity and creating transcriptionally productive enhancers (32, 34). The dependence of cytokine inducibility of

the rAT APRE (in the context of the rAT gene native sequence) on costimulation with glucocorticoids represents a physiologically important demonstration of such an interaction between enhansons which leads to the placement of the rAT gene under dual hormonal regulation. Thus, the rAT gene inducible enhancer unit can be viewed as a genetic-information-processing unit in which the signal conveyed from the IL-1 receptor is gated by glucocorticoids at the level of transcriptional activation of the rAT gene.

The demonstrated interaction between the APRE and an adjacent GRE in the acute-phase activation of the rAT gene is consistent with a model of a direct interaction between their cognate binding proteins (the NFκB-like inducible APRE-binding protein and the glucocorticoid receptor, respectively) or of a synergistic interaction between these proteins and a downstream target. Alternatively, glucocorticoids may enhance accessibility of the APRE-binding protein to its target site, as has been demonstrated for the NF-1 site of the mouse mammary tumor virus promoter (13). In any case, the response appears to be rapid, attained within several hours, and dependent on posttranslational modification of preformed factors.

In terms of its independence from new protein synthesis, the rAT APRE-GRE interaction appears to be different from the mechanism underlying the glucocorticoid dependence of acute-phase induction of the rat α_1 acid-glycoprotein gene (3) or the β-fibrinogen gene (26), inductions which are inhibited by puromycin and cycloheximide, respectively. Acute-phase activation of the α_1 acid-glycoprotein gene appears to share with the rAT gene the potential for glucocorticoid-dependent cytokine activation. When the distant upstream cytokine-responsive element of the α_1 acid-glycoprotein gene is artificially approximated to its proximal GRE (as a result of an internal deletion), the transfected construct assumes a marked degree of glucocorticoid-dependent cytokine activatability [construct pAGP(ΔNdeI-AvaI)CAT; Table 3 in reference 28]. The contribution, however, of a GRE-APRE interaction to the activation of the wild-type α_1 acid-glycoprotein gene, in which the two elements are separated by more than 5 kilobases of intervening sequence, appears to be less (12-fold in the wild-type construct versus 205-fold in the internally deleted construct [Tables 1 and 3 in reference 28]), thus emphasizing the importance of spatial considerations for such interactions between enhansons.

Induction of an NFκB-like APRE-binding protein in HepG2 cells by IL-1 occurs in the absence of glucocorticoid stimulation, and reporter constructs containing multiple copies of the APRE immediately upstream of a minimal promoter are IL-1 inducible in the absence of dexamethasone (30). However, a similarly situated single-copy APRE is only minimally responsive, regardless of glucocorticoid costimulation (unpublished observations). Thus, the rAT APRE exhibits the previously demonstrated enhancer activity of NFκB-binding sites which is dependent on dose and distance from the promoter (27). In this regard, the distant location of the single-copy rAT APRE is in contrast to other inducible promoters which either have multiple NFκB-binding sites, such as the human immunodeficiency virus type 1 long terminal repeat (24) and the serum amyloid A protein gene (16), or have NFκB-binding sites within 100 base pairs of the promoter, as found in the beta-interferon gene (21).

The enhancer activity of NFκB-binding sites in other genes appears to depend also on the adjacent-sequence context, implying the possibility for an interaction with nearby binding proteins. This occurrence is exemplified by the relative inactivity of the NFκB-binding site of the IL-2 receptor alpha gene in phorbol ester-treated fibroblasts or in T lymphocytes (1, 14). In the same experimental system, the NFκB-binding sites of other genes are responsive to such stimuli. In the IL-2 receptor alpha gene, the single-copy NFκB-binding site, located merely 254 base pairs upstream of the transcription start site, already appears to depend on an interaction with adjacent protein-binding sites for its activation (1). An analogous situation may exist in regard to the APRE and GRE in the rAT gene. It appears that contextual constraints of position and adjacent protein-binding sites, rather than properties intrinsic to the rAT APRE, render it dependent on an adjacent GRE for its induction.

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