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MODULATION OF THE AGONIST ACTIVITY OF ANTISTEROIDS BY A NOVEL *cis*-ACTING ELEMENT

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Summary—The amount of agonist activity displayed by the antiglucocorticoid dexamethasone mesylate (Dex-Mes) for the induction of tyrosine aminotransferase (TAT) in rat hepatoma cells is greater than for glutamine synthetase and varies over a period of weeks. This variation, which has been reproduced over a period of 40 h by changing the density of the cells, suggests the involvement of a *trans*-acting factor. The target of this proposed *trans*-acting factor has now been localized to the region between −3.9 to −2.9 of the rat TAT gene from experiments with cells that were stably transfected with hybrid TAT/CAT constructs. Deletion experiments with transiently transfected TAT/tk promoter/CAT constructs revealed that this entire activity could be conveyed by a 21 bp sequence of the TAT gene. Gel shift experiments support the binding of a factor(s) to this 21 bp sequence. Thus the activity of the antagonist Dex-Mes is relatively independent of steroid structure and is largely determined by the further interactions of a *trans*-acting factor with the *cis*-acting sequence. We call this novel sequence a glucocorticoid modulatory element. A model is advanced which accounts for almost all of the results concerning TAT induction by glucocorticoids. This same model may also be useful in explaining why the amount of agonist activity of most antisteroids varies, even for different genes within the same cell.

INTRODUCTION

Antisteroids are usually defined as those compounds which compete with agonist steroids for the binding to steroid receptors and thereby block the action of agonists. An understanding of the mechanism of action of antisteroids would be invaluable in the clinical and basic research setting. Unfortunately, progress in the study of antisteroids has been slow for several reasons. First, given the many steps involved in steroid regulation of gene expression, it is possible that different antisteroids affect different steps [1, 2]. Second, in contrast to agonist steroids, no consistent structure–activity correlations have emerged for antisteroids [3–5]. Third, it has been very difficult to show that pure antisteroids have any intrinsic activity, as opposed to simply preventing the action of agonists. Fourth, virtually all antisteroids show some agonist activity [6]. Fifth, and perhaps most problematic, is the well-known fact that the activity of almost any given antisteroid is not constant. The most famous example of this is tamoxifen, which is full antiestrogen in chicken, a partial antiestrogen in rat, and a full estrogen in mouse [7]. Furthermore, there are numerous examples of the activity of a given antisteroid depending on the gene examined, even in the same cell [5, 6]. Thus the amount of agonist activity expressed by the antiglucocorticoid dexamethasone 21-mesylate (Dex-Mes) [8] for the induction of tyrosine aminotransferase (TAT) vs glutamine synthetase (GS) enzyme activity [9, 10], or TAT vs MMTV RNA [11], is different in Fu5-5 cells. This gene-specific difference in Dex-Mes agonist activity is associated with a difference in the concentration of Dex required for 50% induction (i.e. EC_{50}) [10, 12]. Finally, the gene- and cell-specific differences in amount of Dex-Mes agonist activity, and in Dex EC_{50}, are not constant but change over a period of several weeks in a reversible manner [12]. These observations cannot be explained by the current models of steroid hormone action [13–15], in which the induction of all responsive genes is the same for a given receptor–steroid complex, and exacerbate the current difficulties in understanding the mechanism of antisteroid action.

In an effort to explain the variations in TAT gene expression by Dex-Mes and by subsaturating concentrations of Dex, we have speculated that a *trans*-acting factor binding to a new *cis*-acting sequence may be involved [6; 15a]. The purpose of this paper is to present our evidence

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that such a novel *cis*-acting sequence can determine both the amount of agonist activity seen with an antiglucocorticoid and the EC_{50} of glucocorticoid induction. We have succeeded in isolating such an element from the rat TAT gene and call this sequence a glucocorticoid modulatory element (GME). Finally we discuss a model for GME action that appears to account for all of the known facts about TAT induction. This model, if correct, provides a new approach to understanding the mechanism of antisteroid action.

MATERIALS AND METHODS

Materials

The various chemicals were commercially available from the following suppliers: [^{14}C]-chloramphenicol (57 mCi/mmol, Amersham, Arlington Heights, IL), HEPES (free acid, Ultrol grade, Calbiochem, SanDiego, CA), $CaCl_2$ (Fisher, Pittsburgh, PA), Geneticin (G-418 sulfate, Gibco, Grand Island, NY), Dex and human fibronectin (Sigma, St Louis, MO), phosphate buffered saline (PBS, Mg^{++} and Ca^{++} free, Quality Biological, Rockville, MD), RNase A (from bovine pancreas) and RNase T_1 (Boehringer Mannheim, Indianapolis, IN), restriction enzymes (BRL, Bethesda, MD, and New England BioLabs, Beverly, MA), acetyl CoA (Sigma or Boehringer Mannheim), Hydrofluor (National Diagnostics, Manville, NJ). Dex-Mes was prepared as described [16].

Cell culture, transfection of cells, and selection of stably transfected clones

Monolayer cultures (Fu5-5 cells infected with MMTV (clone 27; Ref. [11]), HTC cells (clone 28; Ref. [12], and transfected cells) were maintained in a 5% CO_2 atmosphere at 37°C in Richter's IMEM and Swim's S77 [both supplemented with 10% heat inactivated fetal calf serum (Biofluids, Rockville, MD) and 0.03% glutamine], respectively.

Stably transfected cells were prepared by the $CaPO_4$ precipitation and G418 selection protocol of Gorman *et al.* [17] as described elsewhere [17a]. Briefly, precipitated plasmid DNA (18 μg of 3.9 or 2.9TATCAT from Dr G. Schütz and 2 μg of RSVNeo) was added to the cells and the mixture was incubated for 16 to 24 h under normal growth conditions (37°C, 5% CO_2 in a humidified incubator) followed by washing, incubation with fresh medium for 24 h, and selection in medium containing the lowest concentration of G418 that kills all the cells within 7 to 10 days (150 μg/ml for HTC cells; 300 μg/ml for Fu5-5 cells). The transformants generated under these conditions were amplified and grown continuously in the presence of G418 supplemented medium in order to maintain the presence of the Neo^r gene.

Construction of plasmids

GREtkCAT, which was originally called PRE-PBL7 and contains 2 repeats of a 23 bp GRE of the rat TAT gene, was obtained from Dr B. O'Malley (Baylor) through Dr J. Ashwell (NIH). Complementary oligonucleotides (20 or 21 bases) were made for O-1 through O-5 and M1 though M4 (Fig. 2) in a DNA synthesizer. After annealing and kinasing, each double-stranded oligonucleotide was ligated in the sense or antisense orientation into the blunt-ended AccI site of the polylinker upstream of the GRE in the GREtkCAT to afford O-1 through O-5 and M1 through M4GREtkCAT.

Transient transfections

Transient transfections were conducted by plating 2×10^6 or 0.3×10^6 cells of Fu5-5 clone 27 or HTC clone 28, respectively, in duplicate 6 cm dishes with 2.5 ml of Richter's IMEM. After 16 h, 3 μg of plasmids and 30 to 35 μg of lipofectin (BRL, Bethesda, MD) were added in 3 ml of Opti-MEM (GIBCO) and cells were incubated at 37°C for 4 h. Fresh Richter's IMEM (2.5 ml) was added and the cells were incubated for 18 h. Cells were then induced for 24 h with the indicated steroid concentrations.

Assay of TAT and CAT activity

Logarithmically growing cells were trypsinized, reseeded, incubated for 24 h at 37°C in fresh medium followed by an additional 20–24 h with fresh medium with or without steroids (ethanol alone, 1 μM Dex-Mes, or 1 μM Dex; final concentration of ethanol = 1%), washed with PBS (4°C), and harvested [18].

For measuring TAT activity, the cell pellets were resuspended in sonication buffer (0.2 mM pyridoxal phosphate, 0.5 mM α-ketoglutarate, 0.1 M potassium phosphate, pH 7.6), lysed by 3 freeze (−80°C)–thaw cycles, heated for 10 min at 65°C, and centrifuged for 15 min at 4°C. The specific TAT enzyme activity (nmol/min/mg protein) in supernatants was then determined [18].

For measuring CAT activity, the procedure of Crabb and Dixon [19] was followed. Briefly, cells were resuspended in 300 μl of Tris buffer (0.25 M Tris, pH 7.8) lysed by 3 freeze (−80°C)–thaw cycles, heated at 65°C for 10 min, and pelleted in a microcentrifuge (15 min at 4°C). After determining the protein content of each supernatant sample (by Bradford assay; BioRad), aliquots containing equal amounts of protein were assayed, analyzed by silica gel chromatography (92.5:7.5, chloroform–methanol), and autoradiographed with Kodak X-OMAT XAR-5 film for 16 h at room temperature. The CAT activity, determined from the dpms in the excised acetylated spots on the TLC plates, was expressed as pmol/min/mg protein.

The activity with Dex-Mes or 4 nM Dex was presented as percent of maximal induction by Dex and calculated as follows: percent Dex-Mes activity = 100 × (induction by 1 μM Dex-Mes–basal activity)/[induction by 1 μM Dex (which is the maximal induction) − basal activity], percent 4 nM Dex activity = 100 × (induction by 4 nM Dex − basal activity)/(induction of 1 μM Dex − basal activity).

Analysis of transcription initiation site by ribonuclease digestions

Total cellular mRNA was purified with oligo-(dT) cellulose (Invitrogen, CA). The template used for generating a T3 antisense RNA probe was constructed by cloning the HindIII–PvuII fragment of GREtkCAT into HincII/HindIII double digested pBluescript KS+ (Stratagene, CA), which was then linearized with EcoRI at −80 bp of the tk promoter. Cellular mRNA (15 μg) was dissolved in 30 μl of hybridization buffer (40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide) and hybridized with 1×10^5 cpm of the uniformly ^{32}P-labeled RNA probe at 48°C for 16 h. The samples were then digested with 300 μl of RNase digestion mixture (2 μg/ml RNase T1 and 40 μg/ml RNase A, 0.3 M NaCl, 10 mM Tris pH 7.4, and 5 mM EDTA) for 1 h at 30°C, processed as described by Melton *et al.* [20], analyzed on 6% urea–polyacrylamide gels, and autoradiographed on the dried gel (Kodak X-OMAT XAR-5 film) for 12 to 24 h at −70°C.

Gel shift assays

Nuclear extracts were prepared as reported [21]. Synthetic oligonucleotides (O-2 or M-2 sequences with SalI cohesive ends) were filled in with Klenow enzyme and labeled with [^{32}P]dCTP. Incubation of the nuclear extracts and the probe was performed as outlined by Weih *et al.* [21] except that 3 μg of extract protein was used and there was no preincubation before adding the probe. The samples were electrophoresed in 5% native polyacrylamide gels in 36 mM Tris–borate buffer (pH 8.4) at constant voltage (10 V/cm) and the gels were dried prior to autoradiography for 12–24 h at room temperature with Kodak X-OMAT XAR-5 film.

RESULTS

Evidence for a cis-acting element that modulates glucocorticoid induction of TAT

If factor-binding to a *cis*-acting element is indeed responsible for the differences in TAT gene expression in Fu5-5 and HTC cells [9, 10], the most likely *cis*-sequence would reside in a DNaseI hypersensitive site that was unequal in Fu5-5 and HTC cells. Recently we have found that of the 12 hypersensitive sites in Fu5-5 cells, the one at −3.6 kb was missing in HTC cells [17a]. Two hybrid TATCAT genes (from Dr G. Schütz) with and without the −3.6 kb hypersensitive site [Fig. 1(A) = 3.9 and 2.9TATCAT] were therefore added to Fu5-5 and HTC cells to prepare stable transfectants in which the endogenous TAT gene would serve as an internal control. In Fu5-5 cells, the ability of Dex-Mes to display levels of agonist activity for CAT induction that were close to those of the endogenous TAT gene was achieved only with cells containing the stably transfected 3.9TATCAT [Fig. 1(B)] [17a]. The percentage of Dex-Mes induced CAT activity in HTC cells containing either TATCAT construct was much lower [Fig. 1(B)] but consistent with the low levels of Dex-Mes agonist activity with the endogenous TAT gene [6, 9, 10, 12, 22]. Thus some element between −2.9 and −3.9 kb of the rat TAT gene appears capable of converting the antiglucocorticoid Dex-Mes to a steroid with high percentages of agonist activity, even for a heterologous gene, in Fu5-5 cells but not in HTC cells. These results also demonstrated that the high levels of Dex-Mes agonist activity for CAT induction in 3.9 vs 2.9TATCAT transfected Fu5-5 cells was not simply a *cis*-acting function of the region of −2.9 to −3.9 kb since the same element was

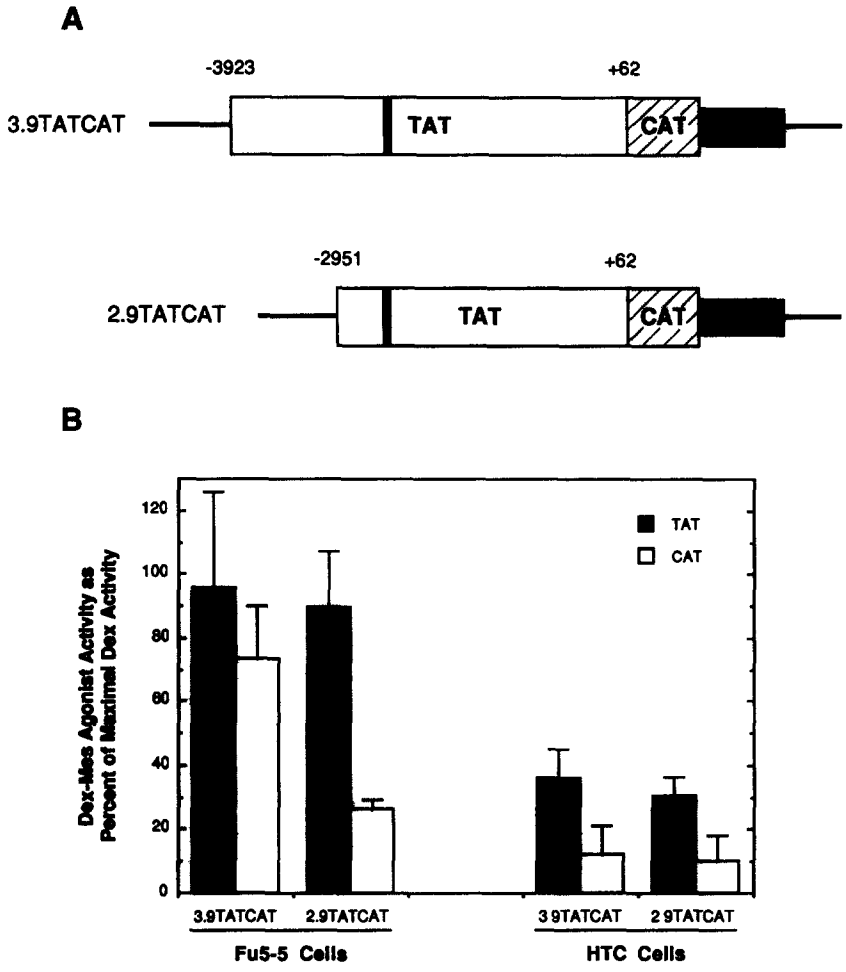

Fig. 1. Induction of CAT enzyme activity from cells stably transfected with hybrid TATCAT constructs. (A) Structures of hybrid TAT/CAT constructs used in preparing stably transfected Fu5-5 and HTC cells. Genomic TAT (open bar), CAT enzyme coding region (hatched bar), SV40 poly A (stippled bar), and GRE (solid bar) sequences are shown along with the plasmid DNA (heavy line). The numbers above the TAT bar correspond to the 5′ and 3′ ends of the genomic TAT sequence in each construct. (B) Percent Dex-Mes agonist activity for induction of TAT and CAT enzyme activity in Fu5-5 and HTC cells stably transfected with 3.9 and 2.9TATCAT. The data are a composite of experiments in which duplicate 60 mm plates of Fu5-5 cells (clone 25 = 3.9TATCAT; clone 11 = 2.9TATCAT) and HTC cells (clone 15 = 3.9TATCAT; clone 20 = 2.9TATCAT) cells were incubated with EtOH, 1 μM Dex, or 1 μM Dex-Mes. The amount of Dex-Mes induced TAT activity (after 16–24 h of incubation, filled bars) or CAT activity (after 20 or usually 24 h of incubation, open bars) was determined and expressed as percent of the maximum induction by Dex (see Materials and Methods). Each bar represents the mean $\pm$ SD for n experiments ($\pm$ range for $n = 2$; the number n for TAT and CAT experiments are listed sequentially after each clone: clone 25 [9, 6]; clone 11 [2, 2]; clone 15 [8, 11]; and clone 20 [2, 2]) (from Ref. [17a]).

ineffective in HTC cells. The maintenance of wild type levels of TAT enzyme expression in the presence of 10 to 14,000 copies of the TATCAT gene [Fig. 1(B)] indicated that there was no "titrating" of limiting transcription factors by the transfected TATCAT constructs [17a]. Finally, we have examined the induction of TAT and CAT in stably transfected cells at different cell densities, conditions which have been found to modulate the amount of Dex-Mes agonist activity with the endogenous TAT gene [22a]. As expected, changes in the density of stably transfected Fu5-5 cells caused a modulation of Dex-Mes induction of CAT activity but only in those cells containing the region between -3.9 to -2.9 kb of the TAT gene [17a]. Collectively, these data argue that the binding of a *trans*-acting factor to a *cis*-acting element between -3.9 to -2.9 kb of the rat TAT gene is responsible both for the higher levels of Dex-Mes agonist activity in Fu5-5 vs HTC cells and for the variation of agonist activity within each cell line.

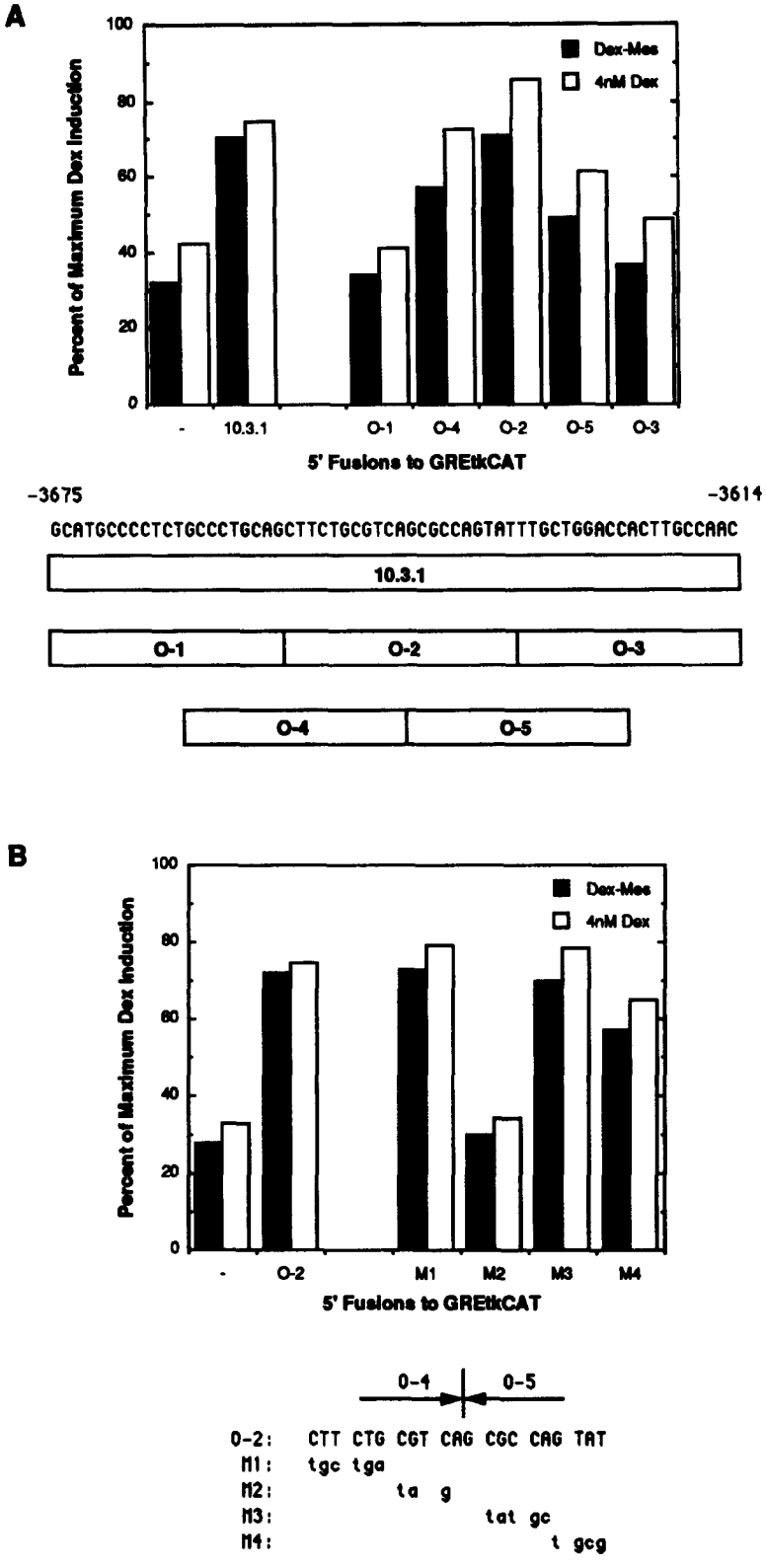

Fig. 2. Identification of the bases required for GME activity. The induction of CAT activity by 1 μM Dex-Mes (solid bars) or 4 nM Dex (open bars), as percent of the maximum induction achieved by 1 μM Dex, from transiently transfected constructs containing either (A) the indicated 62 or 20–21 bp of the TAT gene, or (B) the 21 bp oligonucleotide O-2 with various altered bases, linked to the 5′ end of GREtkCAT, was determined in a representative experiment and plotted as for Fig. 1 (from Ref. [15a]).

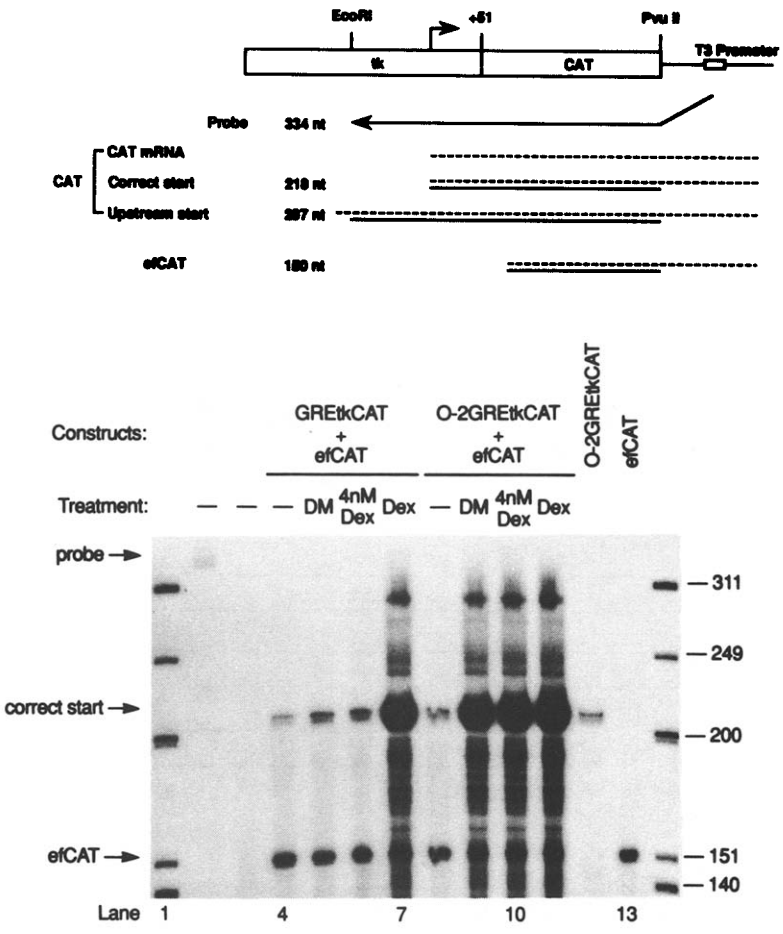

Fig. 3. Effect of GME on the start of transcription of transiently transfected genes in Fu5-5 cells. (top) The expected portions of probe RNA that would be protected from ribonuclease digestion are shown with their calculated size. (bottom) Analysis of start of transcription for transiently transfected GREtkCAT ± O-2. Clone 27 Fu5-5 cells (12.5×10^6 in 15 cm dishes) were transiently transfected with 19 μg of GREtkCAT or O-2GREtkCAT, plus 6.3 μg of the non-inducible efCAT [25] as an internal control, and induced with steroids for 16 h. The mRNA from each treatment was hybridized with uniformly ^{32}P-labeled antisense RNA probe, digested with ribonucleases, analyzed on a 6% urea–polyacrylamide gel, and autoradiographed as described in Materials and Methods. The molecular markers were 5′ ^{32}P-end labeled phiX digested with Hinf I (fragment size is in bases). The positions of undigested probe and correctly initiated transcriptional start sites of GREtkCAT and efCAT constructs are indicated (from Ref. [15a]).

Identification of the cis-acting element that modulates glucocorticoid induction of TAT

In order to further localize the above *cis*-acting element, we turned to transient transfections of hybrid CAT genes containing various deletions of the −3.9 to −2.9 kb region fused upstream of a synthetic glucocorticoid regulatory element (GRE) and the thymidine kinase (tk) promoter (i.e.

(Fig. 4. Opposite)

Fig. 4. Analysis of factor binding to GME by gel shift assays. (A) Competition of binding to O-2 sequence by various oligonucleotides in gel shift assays. The ability of components of Fu5-5 cell nuclear extracts to bind to, and cause a gel-shift of, ^{32}P-labeled O-2 oligonucleotide was examined without (lanes 1,7, and 12) and with a 100-fold excess of various non-radioactive synthetic oligonucleotides [O-1 to O-5, Fig. 2(A), in lanes 2–6 and M1 to M4, Fig. 2(B), in lanes 8–11]. (B) The oligonucleotide sequences of binding sites for other *trans*-acting factors (AP-1 [27], ATF/CREB [28], CRE [29], CREB [30], and USF-II [31]) are listed with the **bold** face letters indicating the homology with the CGTC (in **bold** type) of the O-2 sequence which is required for GME activity. The underlined Gs were determined from methylation interference experiments to be required for factor binding. (C) Ability of oligonucleotides containing the binding site of various *trans*-acting factors to compete for the binding to the GME of O-2. The double stranded oligonucleotides AP1 and CRE (Promega, Madison, WI) and NF-κB, AP2, AP3, and SP1 (Stratagene, LaJolla, CA) were commercially available Only the upper portion of the gel is shown (from Ref. [15a]).

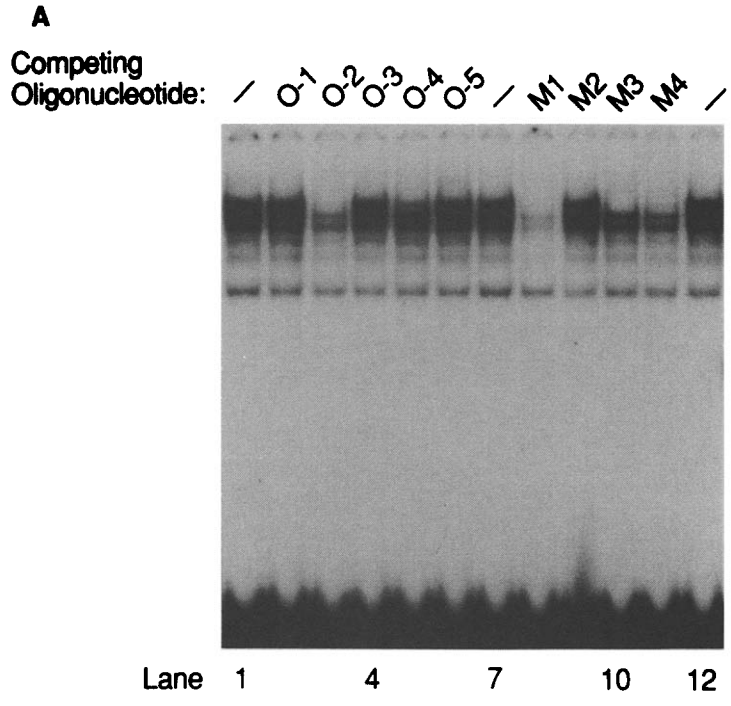

Fig. 4—*legend opposite.*

GREtkCAT). In these studies we also determined the amount of agonist activity displayed by a subsaturating concentration of Dex (i.e. 4 nM Dex).

Previous studies of TAT induction had established a correlation between the amount of agonist activity with 1 μM Dex-Mes and the EC_{50} for Dex [6, 12]. A determination of the percent of maximal agonist activity seen with 4 nM Dex constituted a simplified means of monitoring changes in the Dex EC_{50}. A left shift in the Dex dose–response curve to give a lower Dex EC_{50} would afford higher values for 4 nM Dex agonist activity while lower values with 4 nM Dex would indicate a higher Dex EC_{50}.

A 62 bp fragment (10.3.1) of the TAT gene was eventually identified which gave results in the transient transfection of Fu5-5 cells [Fig. 2(A)] that were very similar to those obtained with the endogenous TAT gene and the larger 3.9TATCAT in stably transfected Fu5-5 cells [cf. Fig. 1(B)]. From these experiments, it was clear that the actions of this *cis*-acting element were not restricted to the TAT gene but could function with both a heterologous gene and a heterologous promoter [15a]. All of the activity of the 62 bp region was found to be contained within the 21 bp fragment O-2 [Fig. 2(A)]. A construct with an inverted O-2 retained >80% activity while 4 or 6 tandem repeats of O-2 were only slightly more active than just one unit of O-2. Induction of a GREtatCAT construct, containing the TAT promoter (−355 to +62 of TAT gene) instead of the tk promoter, was also regulated by the O-2 sequence [15a]. Furthermore, as was seen for the endogenous TAT gene [22a] and for 3.9TATCAT stably transfected in Fu5-5 cells [17a], the amount of Dex-Mes agonist activity in Fu5-5 cells transiently transfected with O-2GREtatCAT could also be varied simply by changing the density of the transfected cells. A similar variation was not obtained if the cells were transfected with GREtatCAT [15a]. Clustered base substitutions of the O-2 sequence indicated that only the 3 bases changed in the M2 mutation were critical for GME activity [Fig. 2(B)]. As expected from our previous results [6, 12], all sequences that afforded increased Dex-Mes agonist activity were equally effective in causing increased 4 nM Dex activity (Fig. 2).

The above effects of the O-2 sequence on glucocorticoid induction were observed at the level of enzyme activity. In principal, different amounts of Dex-Mes, or 4 nM Dex, agonist activity could result from post-transcriptional events or from differential transcription from multiple start sites [23] of the endogenous TAT gene [24]. However, RNase protection assays of CAT mRNA induced in Fu5-5 cells that were either stably transfected with 3.9TAT CAT [17a] or transiently transfected with O-2GREtkCAT (Fig. 3) showed that the same start site was utilized under all conditions. Furthermore, in cells that were transiently transfected with GREtkCAT ± O-2 along with an internal control of efCAT (=pEF-BOS-CAT [25]), the level of correctly initiated transcripts of CAT mRNA exactly paralleled the observed CAT enzyme activities. Therefore we conclude that the GME affects glucocorticoid induction of heterologous or homologous genes by altering the amount of correctly initiated transcripts and probably the rate of transcription [26].

This 21 bp sequence has the properties of an enhancer in that a single O-2 element appears to be sufficient and the activity of the element is relatively position and orientation independent. Because this O-2 element can both control and modulate the amount of Dex-Mes and 4 nM Dex activity induced by a GRE acting on homologous and heterologous genes and promoters, we call this element a glucocorticoid modulatory element (GME).

Evidence for the binding of a trans-acting factor to the GME

Gel shift experiments with ^{32}P-labeled O-2 oligonucleotide and Fu5-5 cell nuclear extracts gave rise to 3 bands [Fig. 4(A)]. The fact that only the top gel-shifted band was specifically competed by those oligonucleotides which displayed high biological activity [i.e. O-2 and O-4 of Fig. 3(A) and M1, M3, and M4 of Fig. 3(B)] argues that the bound protein(s) is the same *trans*-acting factor(s) which binds in whole cells to the GME in the O-2 sequence to afford high levels of Dex-Mes and 4 nM Dex activity [15a]. The observation of a DNaseI hypersensitive site in the TAT gene that encompasses the GME further supports the binding of a factor to the GME [17a].

Comparison of GME with related sequences

Several other factors [Fig. 4(B)] have binding sites which contain the same 4 base sequence that is crucial for GME biological activity

[Fig. 2(B)]. The AP-1 site of Fig. 4(B) shows additional homology; but, an oligonucleotide containing a consensus AP-1 site was unable to inhibit the formation of the biologically relevant top band in the gel shift assay [Fig. 4(C)]. Schütz *et al.* [21, 29, 32] have defined a CRE in the TAT gene that exists at the exact same spot as our GME. However, an oligo containing a consensus CRE site only weakly inhibited the formation of the top band [Fig. 4(C)]. Further comparisons indicated that separate factors bind to the CRE of Schütz *et al.* and to our GME, or at least that the interactions are different. First, the biological activities described for the GME and the CRE are different. Second, CRE, but not GME, activity requires synergism with either another downstream element (BIII at −3602 to −3585 bp) or a tandem repeat of the CRE. Third, the activity of the CRE, but not the GME, is increased upon elevating the intracellular concentration of cAMP with forskolin. Finally, the activity of the native CRE sequence is tissue specific and seen only in liver cells while the activity of the GME was also expressed in non-liver cells (Oshima and Simons, unpublished results).

DISCUSSION

The study of antiglucocorticoid action, and antisteroid action in general, has been complicated by the variety of steps that theoretically can be affected. Current research is focussed around the possibility that the receptor–antagonist complexes bind to HREs but are, for some reason, biologically inactive [33–36]. Explanations for receptor–antagonist inactivity include steroid–receptor interactions which alter the tertiary structure of receptors. New *cis*-acting DNA sequences have been discounted because the only *cis*-acting elements identified so far in the regulation of inducible gene expression are promoters and enhancers [13]. In addition, for "simple" GREs [37], of which the GRE of the TAT gene is the prototype [38], it has been proposed that the sole determinants of glucocorticoid regulation are the GRE and the receptor–steroid complex [38]. However, our studies demonstrate that a new *cis*-acting element is involved in the regulation of TAT gene expression by glucocorticoid agonists and antagonists. In particular, the amount of agonist activity displayed by the antiglucocorticoid Dex-Mes or by subsaturating concentrations of Dex in Fu5-5 cells depends predominantly on the gene examined (e.g. TAT vs MMTV or GS). Furthermore, the amount of agonist activity for TAT induction can be modulated by changing the density of the cells in culture. Both of these effects (increased levels of agonist activity with TAT vs MMTV or GS and the ability to modulate the level of agonist activity for TAT) could be conveyed to a heterologous gene or promoter by a small 21 bp fragment (O-2) located between −3654 and −3634 bp of the rat TAT gene. We call this new element a GME. Gel shift experiments with this O-2 oligonucleotide revealed a presumably protein-bound band, the competition of which was greatest with those oligonucleotides that were the most biologically active. Mutational analysis of the O-2 sequence has identified 4 bases (CGTC) that are required for GME activity. However, other bases are also important since this same 4 base sequence is present in the DNA binding sequences of several other *trans*-acting factors, none of which have the same properties or biological activity as the GME. Thus we conclude that components other than the receptor–steroid complex, the GRE, and the promoter can both regulate glucocorticoid induction of a responsive gene and determine the activity of an antisteroid.

The current models of steroid hormone action do not include any *cis*-acting elements such as the GME [13–15]. On the other hand, the current models are also incapable of explaining the observed variations in agonist activity of antisteroids or subsaturating concentrations of agonists. In order to explain such modulation, we propose a model (Fig. 5) that incorporates the GME and its binding of a *trans*-acting factor (F) along with four other documented features of receptors: (1) the ability of receptor–antisteroid complexes to bind to HREs [33–36]; (2) the capacity of DNA-bound receptor–steroid complexes to interact with general and gene-specific proteins [39–42]; (3) the differential effects of agonists and antagonists on the transcriptional activation activity of two different domains of the receptor protein, TAF1 and TAF2 [1, 43, 44]; and (4) a mathematical explanation for left shifts in the EC_{50} of steroid receptor mediated gene expression [6, 45]. A crucial feature of our model is the GME-binding *trans*-acting factor, the levels or activities of which appear to be regulated in the cell. The model also requires that the production of the induced gene transcript follows an ordered

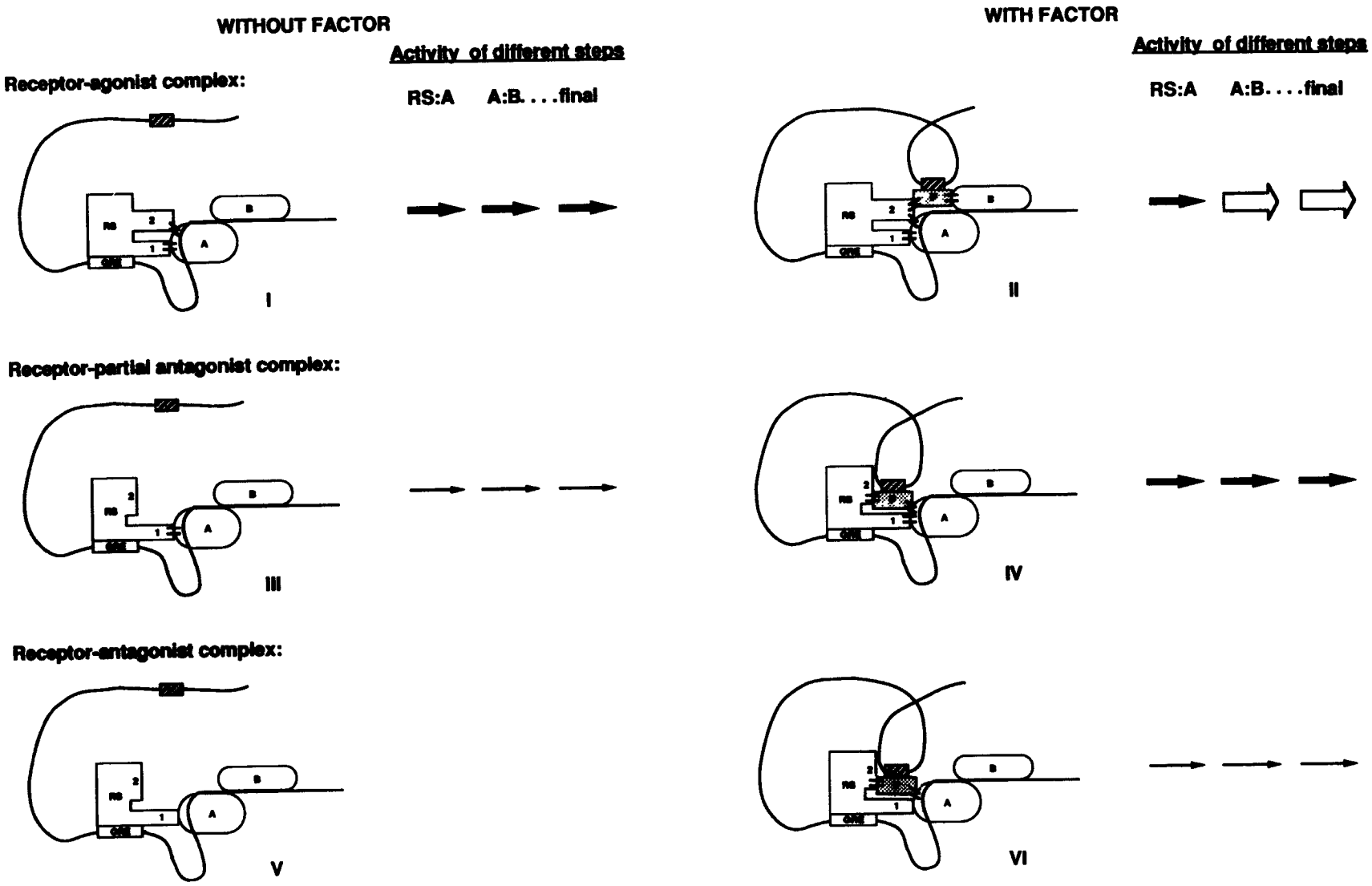

Fig. 5. Model for action of GME in modulation of glucocorticoid induction of TAT gene expression. The induction of a general glucocorticoid-regulated gene product is postulated to result from the combined interaction of GRE-bound receptor–steroid complexes (RS) with transcription factors A and B and the proposed *trans*-acting factor (F), which binds to the GME (diagonally striped box). A series of coupled steps or interactions are thought to be required to give the eventually observed induction (or repression) of the regulated mRNA. Whether transcription activation domains 1 and 2 of the RS complex are active depends on the nature of the bound steroid (see text for details) (from Ref. [15a]).

series of steps, commencing with the interaction of receptor–steroid complex with factor A. While other scenarios may be formulated within these constraints, we propose that modulation of glucocorticoid regulated gene expression could occur as follows. The GME-bound factor (F) would interact both with a *trans*-activating domain of the receptor (e.g. TAF2) and with transcription factors A or B. For complexes with agonists (I of Fig. 5), interaction of the GME-bound factor F to give II would increase the efficiency of coupling in the next step required for induced transcription (i.e. an interaction between A and B, which is represented by the second arrow in "activity of different steps"). It is this increased coupling efficiency at subsaturating concentrations of steroid that causes a left shift in the dose–response curve [6, 45]. For partial (III) and full (V) antagonists, TAF2 region is altered [1, 43, 44] and the interactions seen for full agonists are not possible. However, if the factor-binding site of TAF2 is intact, a complex (IV or VI) could be formed that contained interactions similar to those for TAF2 in the agonist complex. In this manner, the amount of agonist activity for partial and full antagonists would be increased. In all of the above situations, alterations in the intracellular levels or activities of factor F would affect the responses both to agonists (to shift the dose–response curves) and to partial and full antagonists (to alter the amount of agonist activity). Clearly this is a speculative model which needs extensive testing. However, this is the only model that we have seen which can account for all of the present observations on antisteroid activities.

In summary, the activity of antiglucocorticoids is not constant but can vary with the gene examined. For the TAT gene, this variation is dictated by a *cis*-acting element which resides between −3654 and −3634 bp of the rat TAT gene. We call this element a GME. While this GME has the properties of a classical enhancer element (if acts at a distance, in either orientation, and influences heterologous genes and promoters), the activity of this GME is unlike that of any reported transcriptional element of which we are aware. We have advanced a model, containing this GME, which can account for the previously unexplained ability of antagonists to exhibit variable amounts of agonist activity, and for agonists to have different EC_{50}s, for the same or different genes in the same cell. Furthermore, a survey of the literature suggests that the presence of a GME is not limited to the TAT gene but may be involved in the regulation of other genes both by glucocorticoids and by other steroid hormones [5]. Thus these results with the GME provide an expanded framework for examining the general mechanism of steroid hormone action. Furthermore, the TAT gene system constitutes a more defined system for studying antisteroid action and may finally yield consistent structure–activity relationships for antisteroids.

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