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Glucocorticoid Receptor Binding to a Specific DNA Sequence Is Required for Hormone-Dependent Repression of Pro-Opiomelanocortin Gene Transcription

JACQUES DROUIN,[1]* MARK A. TRIFIRO,[1] RICHARD K. PLANTE,[1] MONA NEMER,[1] PER ERIKSSON,[2] AND ÖRJAN WRANGE[2]

Laboratoire de Génétique Moléculaire, Institut de Recherches Cliniques de Montréal, 110 Avenue des Pins Ouest, Montreal, Quebec, Canada H2W 1R7,[1] and Department of Molecular Genetics, Karolinska Institutet, S-104 01 Stockholm, Sweden[2]

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Glucocorticoids rapidly and specifically inhibit transcription of the pro-opiomelanocortin (POMC) gene in the anterior pituitary, thus offering a model for studying negative control of transcription in mammals. We have defined an element within the rat POMC gene 5′-flanking region that is required for glucocorticoid inhibition of POMC gene transcription in POMC-expressing pituitary tumor cells (AtT-20). This element contains an in vitro binding site for purified glucocorticoid receptor. Site-directed mutagenesis revealed that binding of the receptor to this site located at position base pair −63 is essential for glucocorticoid repression of transcription. Although related to the well-defined glucocorticoid response element (GRE) found in glucocorticoid-inducible genes, the DNA sequence of the POMC negative glucocorticoid response element (nGRE) differs significantly from the GRE consensus; this sequence divergence may result in different receptor-DNA interactions and may account at least in part for the opposite transcriptional properties of these elements. Hormone-dependent repression of POMC gene transcription may be due to binding of the receptor over a positive regulatory element of the promoter. Thus, repression may result from mutually exclusive binding of two DNA-binding proteins to overlapping DNA sequences.

Glucocorticoid hormones modulate expression of many genes, and both glucocorticoid-inducible (reviewed in references 5 and 69) and glucocorticoid-repressed (7, 11, 13, 20, 22–24, 30, 31, 35, 66) genes have been described. The mechanism responsible for glucocorticoid induction of transcription was studied in detail after the localization of a glucocorticoid response element (GRE) in the promoter region of the mouse mammary tumor virus (MTV) genome (9, 33, 38, 39) and in several other glucocorticoid-inducible genes (5, 69). Transduction of the hormonal signal is mediated through specific binding of the glucocorticoid receptor (GR) to this regulatory element (6, 48, 60). How interaction of the receptor with the conserved GRE sequence leads to activation of transcription and what other promoter elements may be required for this activation remain unclear (15, 56, 61). In any event, GREs behave as hormone-dependent enhancer elements acting over variable distances and in either orientation (12, 51). Although this enhancerlike activity can account for transcription stimulation, it is difficult to reconcile with glucocorticoid-dependent repression of transcription.

Molecular events involved in repression of transcription have been analyzed in bacterial and yeast cells (8, 53) but remain poorly understood in mammalian systems. Although repression of transcription by steroid hormones has been documented in a few systems (3, 11, 13, 22, 23, 30, 66), the role of receptor-DNA interactions in this regulatory mechanism remains unclear (1, 3, 31, 58). The negative-feedback action of glucocorticoids on pro-opiomelanocortin (POMC) gene transcription provides a relevant system for investigation of this mechanism, since negative-feedback regulation between adrenal glucocorticoids and pituitary adrenocorticotropin production is one of the oldest known physiological feedback loops. The inhibition of POMC gene transcription by glucocorticoids is specific to adrenocorticotropin-producing cells of the anterior pituitary and does not affect melanotropin-producing cells of the intermediate pituitary lobe (7, 20, 21, 23, 24).

Previous work has shown that POMC promoter sequences are responsible for glucocorticoid regulation of both the rat (13) and human (35) genes. We have recently defined rat POMC promoter sequences required for tissue-specific expression in AtT-20 pituitary tumor cells (36) and shown that these sequences confer pituitary-specific expression and glucocorticoid regulation in transgenic mice (63). We now report the localization within this promoter region of a negative GRE (nGRE) that binds the GR in vitro. Binding of the receptor to this element appears essential for repression of POMC gene transcription by glucocorticoids.

MATERIALS AND METHODS

Plasmids. Recombinant plasmids were constructed by standard procedures (40) and purified by banding twice on cesium chloride gradients. Construction of the various 5′-deletion mutants of the POMCneo plasmids was described elsewhere (36). The length of POMC 5′-flanking sequences present in each plasmid construct is indicated by a subscript in the plasmid name. In addition to plasmid $pPOMC_{706}neo$, the following pBR327 subclones of rat POMC genomic sequences were used in footprinting experiments: pJA21, which contains a 4.5-kilobase-pair (kb) *Eco*RI-*Bam*HI fragment (Fig. 1); pJA11, which contains a 2-kb *Bam*HI fragment (Fig. 1); and pJA55, which contains the 769-base-pair (bp) *Xmn*I POMC promoter fragment (enlarged in Fig. 1) subcloned between the *Hin*dIII and *Sal*I sites of pBR327 by using synthetic linkers. Probes for GR footprinting of MTV

* Corresponding author.

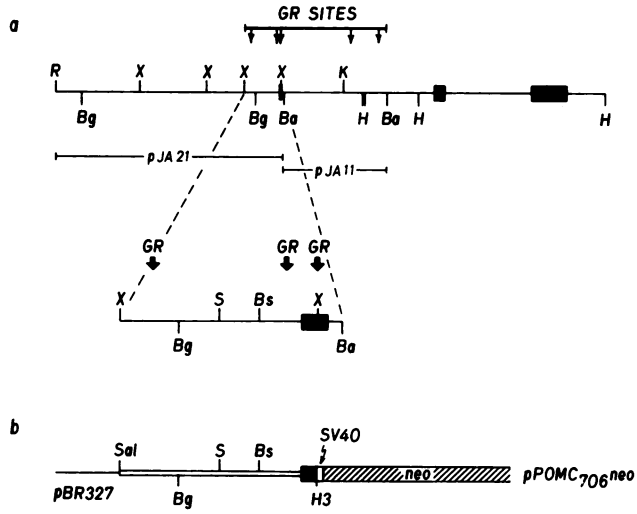

FIG. 1. GR-binding sites in the rat POMC gene. (a) Positions of GR-binding sites identified in vitro on a restriction map of the rat POMC gene (18); POMC transcription is from left to right, and the three POMC exons are indicated (■). The line at the top indicates the 3-kb region that was screened for GR-binding sites by DNase I footprinting; arrows indicate positions of the five GR-binding sites within this region. The 5′ region of the gene (16) is enlarged to show the two GR-binding sites in 5′-flanking sequences as well as the low-affinity site in the first exon. Abbreviation for restriction enzyme sites: R, *Eco*RI; H, *Hin*dIII; Ba, *Bam*HI; Bg, *Bgl*II; X, *Xmn*I; K, *Kpn*I; S, *Stu*I; Bs, *Bss*HII. (b) Structure of the hybrid POMCneo transcription unit used in electroporation experiments for plasmid $pPOMC_{706}neo$. This plasmid contains a rat POMC promoter fragment extending from positions −706 to +63 in the first exon. As indicated, a 24-bp fragment from the 5′ untranslated region of the simian virus 40 (SV40) early transcription unit (*Hin*dIII-to-*Stu*I fragment) is inserted between POMC promoter sequences and the bacterial neomycin resistance gene. The simian virus 40 sequences were used in RNase mapping experiments as a tag to differentiate endogenous mPOMC mRNA from rPOMCneo mRNA transcripts.

sequences were derived from plasmid pLS5′139, which contains MTV long terminal repeat (LTR) sequences from positions bp −263 to −109 fused to herpes simplex virus thymidine kinase gene sequences at position bp −107 (67).

For site-directed mutagenesis, the 793-bp *Sal*I-*Hin*dIII POMC promoter fragment from $pPOMC_{706}neo$ was subcloned into M13mp18 to generate single-stranded templates. The double point mutation was introduced in the POMC promoter with a 20-nucleotide (nt) synthetic oligonucleotide as described previously (44), using kits from Amersham Corp. (Arlington Heights, Ill.). Promoter fragments containing the expected mutation were identified by DNA sequencing and transferred into plasmid $pPOMC_{480}neo$.

Receptor footprinting. DNase I footprinting was performed as described previously (48), using purified rat liver GR (68). In some experiments, the last DEAE-Sepharose step was replaced by chromatography on fast protein liquid chromatography (FPLC) Mono Q (Pharmacia, Uppsala, Sweden) (49). Dimethyl sulfate (DMS) methylation protection experiments were performed as described previously (59). The procedure of von der Ahe et al. (64) was used in exonuclease III footprinting experiments except that a concentration of 5 instead of 2 mM $MgCl_2$ was used. DNA fragments were labeled either at the 5′-end with T4 polynucleotide kinase or at the 3′ end with the Klenow fragment of *Escherichia coli* DNA polymerase I and ^{32}P-labeled triphosphates (40). In footprinting and DMS protection experiments, about 30,000 cpm of ^{32}P-labeled DNA fragments (0.2 to 5 ng) was incubated with the indicated amounts of purified GR. The receptor was quantitated by assuming the existence of one hormone-binding site per molecule (68).

Gene transfer. AtT-20/D16-v cells (obtained from R. Kelly) were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum. Electroporation was performed as described previously (52), using 5×10^6 cells and supercoiled plasmid DNAs at a concentration of 50 μg/ml. After 2 weeks of selection in the presence of 750 μg of G418 (Geneticin; GIBCO Laboratories, Grand Island, N.Y.) per ml, foci were counted, pooled, and propagated in mass culture for analysis of glucocorticoid sensitivity. In most cases, more than 100 G418-resistant colonies were pooled for analysis, and three to four different pools from different electroporation experiments were assayed for glucocorticoid sensitivity. To test for glucocorticoid sensitivity, control and electroporated AtT-20 cells were grown for 2 days in Dulbecco modified Eagle medium containing 10% fetal calf serum, which was stripped of endogenous steroids by absorption with dextran-coated charcoal (19). Cultures were then treated overnight in the same medium containing 10^{-7} M dexamethasone (Dex), 10^{-6} M RU-38486, or solvent.

RNA analysis. Total cytoplasmic RNA was extracted from cells as described previously (14). The 5′ ends of rat POMCneo (rPOMCneo) and mouse POMC (mPOMC) mRNA transcripts were quantitated by densitometry of appropriate bands in RNase mapping experiments, using an RNA probe produced on an SP6 template (41) as described previously (13). Cellular β- and γ-actin mRNAs were quantitated by Northern (RNA) blot in parallel with the RNase mapping to serve as internal control; a 1,500-bp rat brain β-actin cDNA fragment (supplied by S. Farmer, Boston University School of Medicine, Boston, Mass.) was used as a probe. For the point mutation experiment (see Fig. 7), rPOMCneo, mPOMC, and β- and γ-actin mRNAs were quantitated by densitometric analysis of Northern blots sequentially hybridized with the three appropriate probes. In all experiments, Dex inhibition of rPOMCneo and mPOMC mRNA transcripts was corrected for β- and γ-actin mRNA levels, which were not affected by Dex and served as an internal control.

For S1 nuclease mapping (40) of RSVneo transcripts, 5 μg of cellular RNA was hybridized overnight at 52°C with 100,000 cpm of ^{32}P-labeled probe. The 325-bp DNA probe was labeled with T4 polynucleotide kinase at a *Nar*I site within *neo* sequences and produced by recutting at an *Eco*RI site located at position bp −50 within the Rous sarcoma virus (RSV) LTR. S1 nuclease digestion of hybrids between this probe and RSVneo transcripts should therefore result in protection of a 275-nt fragment. This fragment was indeed the only one observed in these experiments (see Fig. 6). Northern blot analysis of β- and γ-actin mRNA transcripts was used to correct for slight variations among samples.

RESULTS

GR-binding sites. Glucocorticoid induction of transcription is mediated by direct GR contact with DNA. To determine whether glucocorticoid inhibition of POMC transcription is associated with binding of the GR to specific sequences in the POMC gene, we analyzed the binding of purified rat liver GR to rat POMC genomic DNA. The FPLC-purified receptor preparations used in these studies were at least 95% pure, as judged by silver staining of polyacrylamide gels (49, 68). MTV promoter fragments

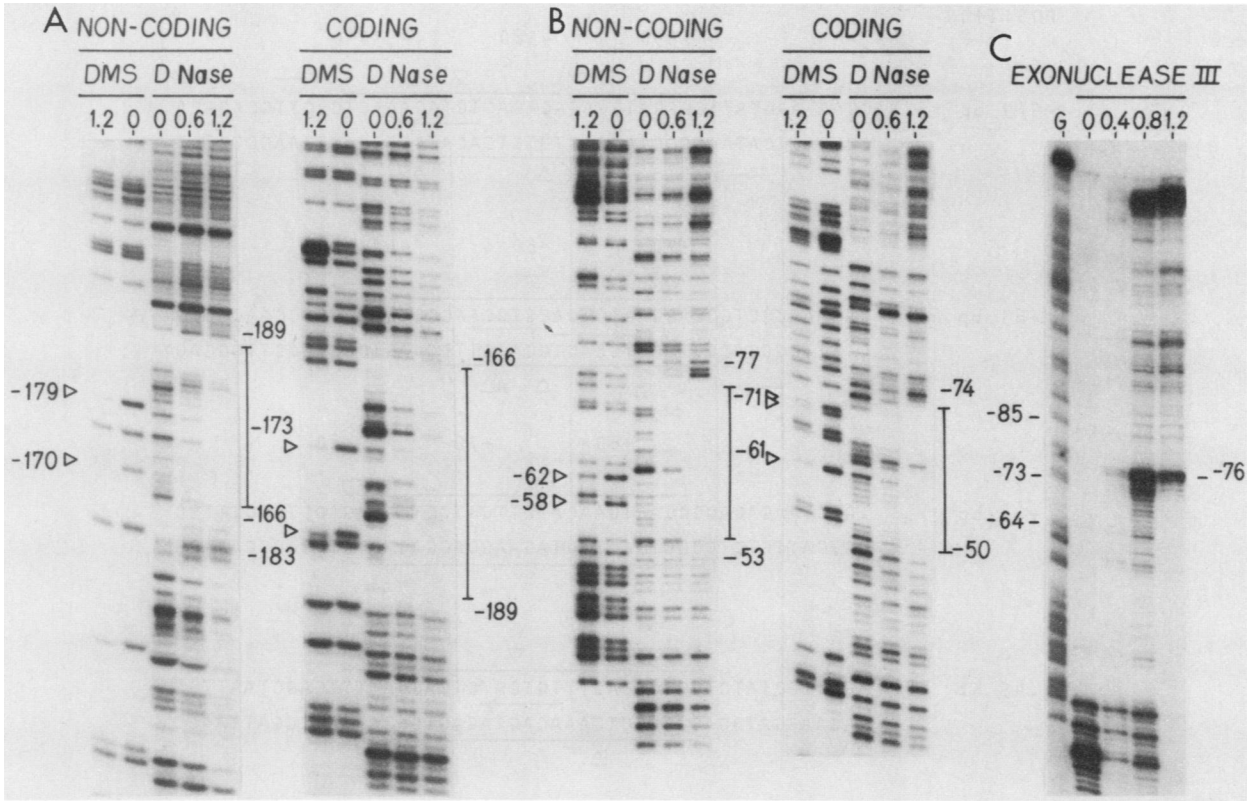

FIG. 2. Footprint analysis of POMC and MTV GR-binding sites. GR binding was carried out in parallel on both coding and noncoding DNA strands of the MTV LTR (A) and the POMC promoter (B and C). Receptor binding was analyzed by DNase I footprinting and by DMS protection (A and B) and by exonuclease III footprinting (C) in the presence of increasing amounts (indicated at the top in picomoles) of purified rat liver GR. Positions of GR-dependent DNase I footprints are indicated on the sides by vertical bars relative to sites of MTV (A) and POMC (B) gene transcription initiation. Guanosine residues protected from DMS attack in the presence of receptor are also indicated (▷). (A) DNA probes for coding and noncoding strands of the MTV LTR sequences were obtained by 5'-end labeling at the *Sst*I (position bp −263 of the MTV gene) and *Eco*RI (position bp −80 of the thymidine kinase gene) sites, respectively, of plasmid pLS5'139. (B) DNA probes for coding and noncoding strands of the POMC promoter were obtained by 3' and 5' end labeling, respectively, at the *Sal*I site (position bp +63 of the POMC gene) of plasmid pJA55. (C) A 253-bp *Bss*HII-*Hin*dIII fragment isolated from pPOMC$_{706}$neo and 5' end labeled at the *Hin*dIII site was used in an exonuclease III footprinting experiment.

containing GR-binding sites were used as a control (Fig. 2A) in DNase I footprinting experiments (25). Four POMC DNA segments, each 23 to 26 bp long, showed GR-dependent protection on both DNA strands at receptor concentrations similar to or slightly higher than those required for protection of the MTV GRE (Fig. 1 to 3). At much higher receptor concentrations, a fifth site was observed within exon 1 at position bp +64. Since this site had a much lower affinity for GR, it was not studied further. Two GR-binding sites are located in the 5'-flanking region (they are centered on positions bp −579 and −63), and the other two are in the first intron (at positions kb +1.45 and +1.9). It was previously shown that exons 2 and 3 and intron B of the rat POMC gene do not contain GR-binding sites (48). Thus, five GR-binding sites, including the low-affinity site, are present in the rat POMC gene. The localization of the 3' borders of these sites was refined in exonuclease III protection experiments (64). The results of these experiments are in agreement (within 2 to 3 bp) with the borders of the footprints detected by DNase I digestion (Fig. 2C and 3). It should be mentioned that receptor preparations made according to the published procedure (68) and without the FPLC purification step produced an additional footprint at position bp −146 in the POMC promoter (17); this footprint was not observed with FPLC-purified receptor, and its sequence does not show any homology to those of the other footprints.

The interaction of the GR with POMC gene binding sites was further characterized by DMS protection experiments (46). In this assay, guanine residues making major groove contact with the GR were protected from DMS methylation. In MTV and in other glucocorticoid-inducible genes (59), binding of the GR resulted in protection of specific guanine residues from DMS methylation, in particular within the conserved hexanucleotide sequence 5'-TGTTCT-3', in which guanine residues on both strands were protected (Fig. 2A and 3). A homologous hexanucleotide sequence and a similar pattern of DMS hypomethylation were found within POMC gene GR-binding sites (Fig. 2B and 3). Whereas the bp −63 site contained two DNA sequences on opposite strands that were homologous to the consensus hexanucleotide (indicated by arrows in Fig. 3), the DMS protection pattern identified the coding-strand hexamer as the one having intimate contact points with the receptor; therefore, this hexamer was used for sequence alignment and was taken as a reference to infer receptor recognition of this binding site.

Glucocorticoid inhibition of transcription. Glucocorticoids decrease anterior pituitary POMC mRNA by a specific,

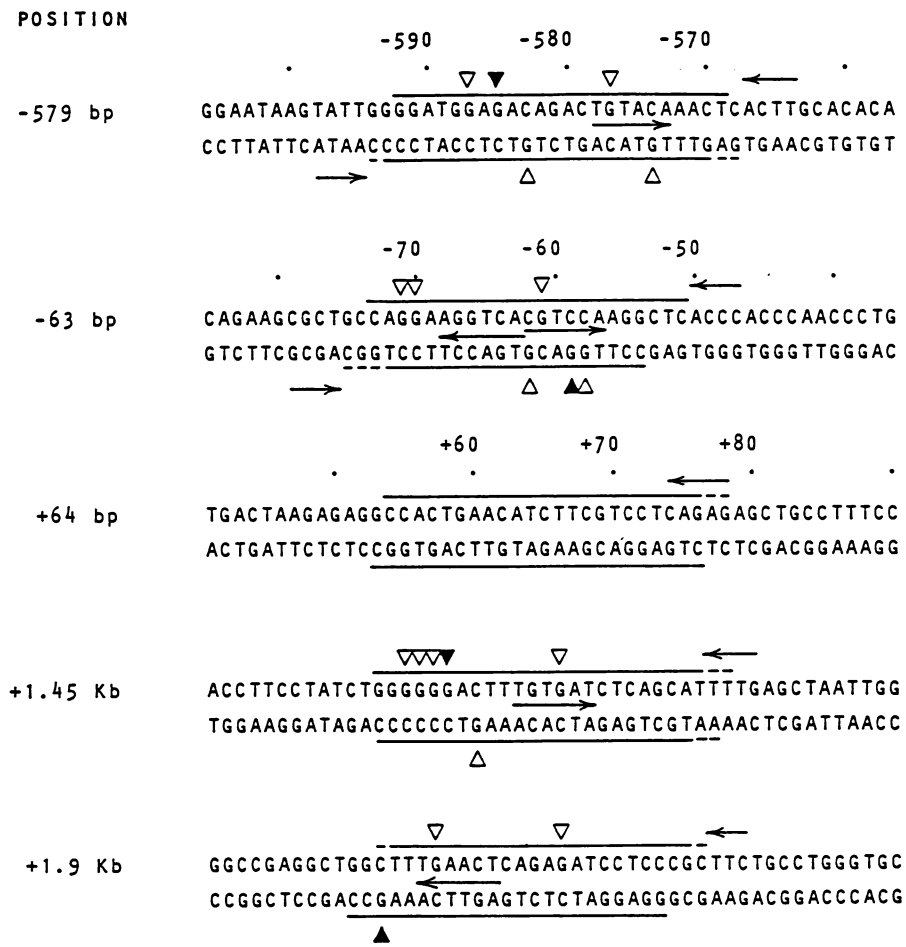

FIG. 3. DNA sequence of the five POMC GR-binding sites. Each GR-binding site is identified by the position of the central nucleotide in the DNase I footprint. Lines above and below the DNA sequences indicate nucleotides that were protected on each strand from DNase I digestion in the presence of receptor. Arrows above and below sequences indicate the 3′ borders of receptor-binding sites as determined by exonuclease III footprinting experiments. Guanine residues that were undermethylated (▽) and overmethylated (▼) by DMS in the presence of receptor are indicated. Arrows between DNA strands indicate hexanucleotides that are homologous to the consensus 5′-TGTYCT-3′ present in most GR-binding sites described to date (5).

rapid, and protein synthesis-independent action at the transcriptional level (23, 24). To identify POMC sequences responsible for glucocorticoid inhibition of transcription, we constructed chimeric genes containing different rat POMC promoter fragments fused to coding sequences for bacterial neomycin resistance (36). Structures of the chimeric POMCneo genes are illustrated in Fig. 1b. In addition to containing 5′-flanking sequences, all POMC promoter fragments extended to nucleotide +63 within the first exon. These exon sequences contained only half of the bp +64 GR-binding site, and they no longer bound the receptor in DNase I footprinting experiments (data not shown). To assess glucocorticoid regulation, POMCneo plasmids were introduced by electroporation into AtT-20 mouse tumor cells. This cell line is an ideal model for studies of POMC expression, since the POMC gene is expressed and regulated in these cells as in anterior pituitary corticotrophs. In particular, transcription of the gene is inhibited by glucocorticoids and stimulated by corticotropin-releasing hormone (2, 45, 57).

Using AtT-20 cells and various POMCneo plasmids, we have previously shown that no more than 480 bp of 5′-flanking POMC sequences is required for tissue-specific expression (36). We have now used the same pools of electroporated AtT-20 cells to assess glucocorticoid regulation of POMCneo transcripts. An RNase protection assay that allows simultaneous detection of endogenous mPOMC and rPOMCneo mRNA transcripts was used to quantitate the inhibitory effect of the synthetic glucocorticoid Dex. The structure of the RNA probe used in these assays is illustrated in Fig. 4B; as indicated, hybridization of this probe to mPOMC and rPOMCneo mRNA transcripts should protect fragments of 63 and 87 nt, respectively. The ladder of bands observed under the 63-nt fragment resulted from breathing of the imperfect hybrid between mPOMC mRNA and the rat POMC RNA probe and depended on the extent of RNase digestion.

Initially, two large POMC promoter fragments (bp −4500 and −706) were tested for glucocorticoid inhibition, since we had previously shown by using a viral vector system that the 706-bp promoter fragment conferred glucocorticoid inhibition (13). Dex treatment of AtT-20 cells electroporated with these plasmids indicated that both endogenous mPOMC and rPOMCneo mRNA transcripts were decreased about threefold by the hormone, as assessed by densitometry of RNase mapping autoradiographs and correction for β- and γ-actin

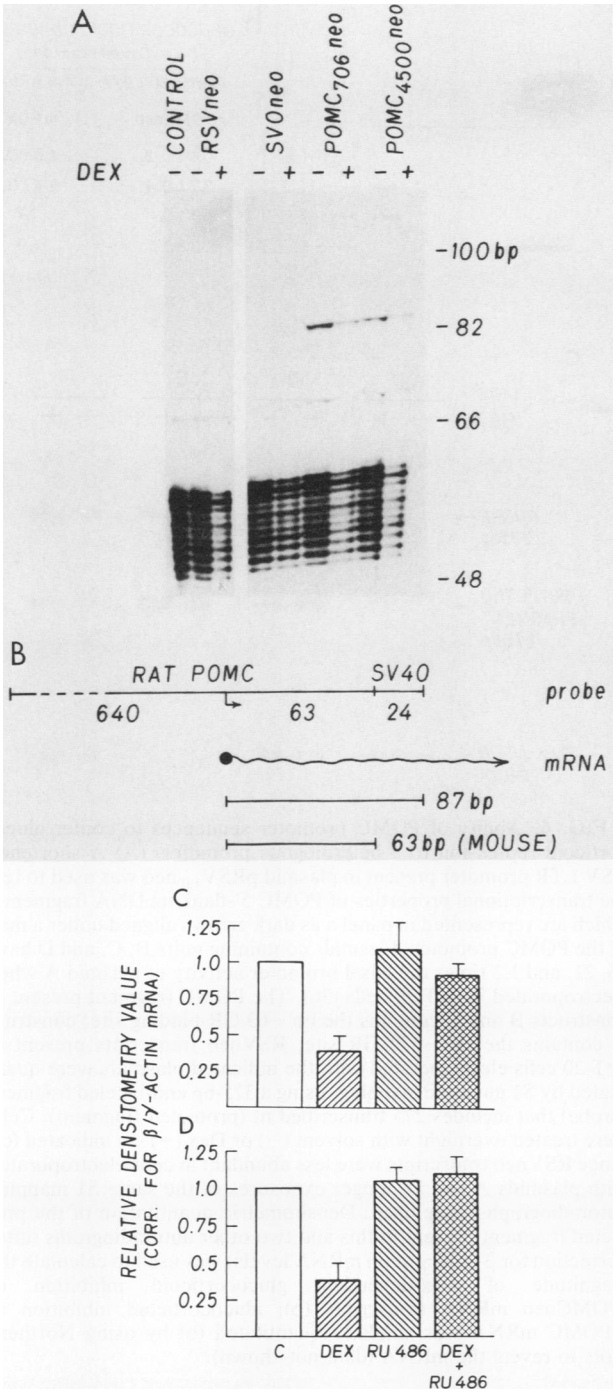

FIG. 4. Ability of POMC fragments to confer glucocorticoid repression. (A) RNase mapping assay measuring POMC gene transcripts in total RNA extracted from control AtT-20 cells or pools of G418-resistant AtT-20 cells electroporated with the indicated plasmids. (B) Structure of the RNA probe used to detect the 5′ ends of both endogenous mPOMC and rPOMCneo mRNA transcripts in electroporated AtT-20 cells. In the RNase mapping assay, the probe produced the expected 63-nt fragment by hybridization to the mPOMC mRNA present in control AtT-20 cells (first lane). The same probe also produced an 87-nt fragment when hybridized to RNA samples from cells electroporated with plasmid $pPOMC_{4500}neo$ or $pPOMC_{706}neo$; this fragment is expected from protection of correctly initiated rPOMCneo mRNA transcripts. Pools of G418-resistant AtT-20 cells were incubated in the absence (−) or presence (+) of Dex. (C and D) Prevention of Dex repression by the antiglucocorticoid RU-38486. AtT-20 cells electroporated with plasmid $pPOMC_{480}neo$ were incubated overnight with 10^{-7} M Dex, 10^{-6} M RU-38486 (RU486), both, or solvent (C). The levels of rPOMCneo (87-nt RNase mapping fragment) (C) and mPOMC mRNA (63-nt RNase mapping fragment) (D) were measured in total RNA extracted from treated cells, using the RNase mapping assay, densitometry, and correction for the levels of β- and γ-actin mRNAs quantitated on Northern blots. The data (means and standard deviations of three determinations) are presented in arbitrary densitometry units relative to values for the control (column C) sample.

levels (Fig. 4). Also, the two POMC promoter fragments conferred similar inhibition, indicating that no more than 706 bp of the promoter was required for glucocorticoid repression. To assess the role of the GR in this repression, the effect of the antiglucocorticoid RU-38486, which is known to act at the receptor level in glucocorticoid-inducible systems (10, 43), was tested. RU-38486 completely prevented the inhibition of both mPOMC and rPOMCneo mRNA transcripts by Dex (Fig. 4C and D), indicating that this response is receptor mediated.

To further localize the nGRE within the POMC promoter, a series of 5′-deletion mutants was tested for glucocorticoid inhibition. Deletion of POMC sequences up to position bp −166 did not interfere with glucocorticoid inhibition (Fig. 5), although promoter activity is progressively reduced when sequences beyond position bp −480 are deleted (36). These results indicate that the GR-binding site present at position bp −579 is not essential for glucocorticoid inhibition, since its deletion did not affect the magnitude of Dex repression.

Further resection of the promoter to position bp −132 affected its activity so much that glucocorticoid sensitivity could not be tested. To determine whether the bp −63 GR-binding site is required for glucocorticoid inhibition, we deleted this site from a POMC promoter fragment extending to position bp −480. Deletion of sequences between positions bp −132 and −34, which eliminated the bp −63 binding site, abolished glucocorticoid repression (Fig. 5). Conversely, deletion of sequences between positions bp −166 and −132 did not prevent glucocorticoid inhibition. Thus, a POMC promoter fragment extending from positions bp −132 to −34 and containing a single in vitro binding site for the GR appears essential for glucocorticoid inhibition of transcription. We tested whether this fragment could confer glucocorticoid inhibition to a heterologous promoter by fusion of this fragment upstream of a shortened RSV promoter. As described earlier (36), insertion of this POMC fragment in either orientation increased promoter activity 10- to 20-fold and resulted in higher levels of RSVneo transcripts, as measured in S1 nuclease mapping experiments (Fig. 6, constructs B and C as compared with construct A). In addition, this POMC fragment conferred glucocorticoid inhibition to the RSV promoter when inserted in the reverse orientation (Fig. 6). The reason for this orientation dependence is not immediately obvious but may reflect the importance of position within the promoter for nGRE activity. Of all other POMC promoter fragments tested (data not shown), only one conferred weak repression (about 1.5-fold); this fragment is the same as construct B but it extends to position bp −166 and thus has an added 34 bp between RSV and POMC sequences. A construct containing the bp −579 GR-binding site (Fig. 6, construct D) conferred neither induction nor repression of glucocorticoid synthesis.

Receptor binding is correlated with repression. The necessity for GR binding to the bp −63 site in transduction of the hormonal signal to POMC gene transcription was assessed

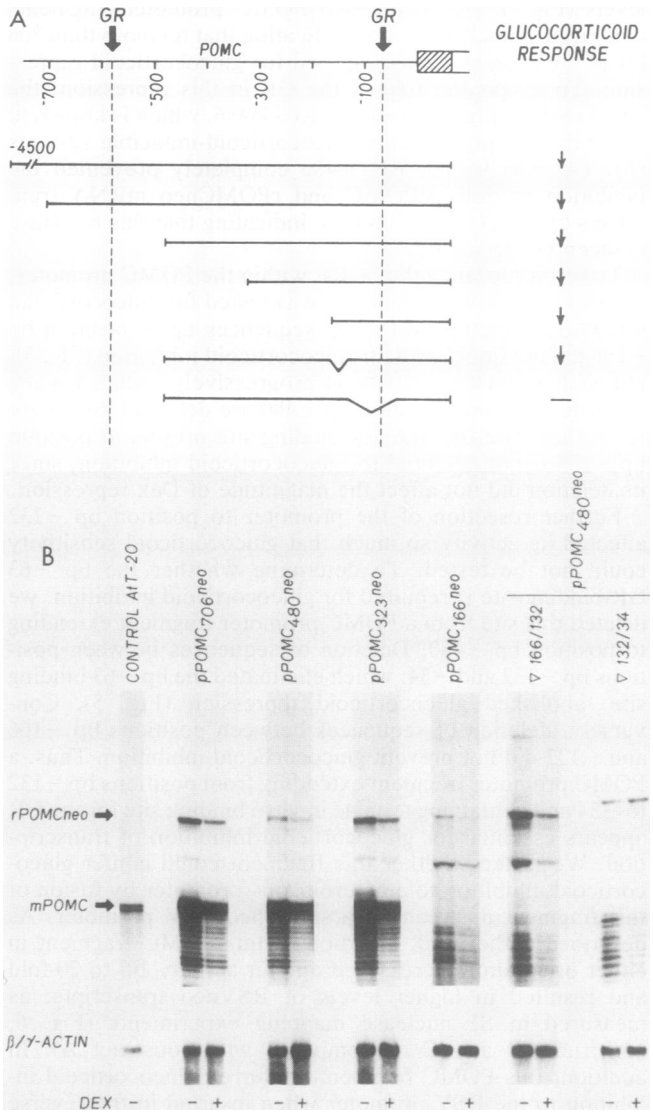

FIG. 5. Localization of the POMC nGRE. POMC promoter deletion mutants were used to localize sequences required to confer glucocorticoid repression of transcription. (A) The glucocorticoid response of various 5'-deletion and internal-deletion mutants represented schematically below a map of the rat POMC promoter in which in vitro GR-binding sites are indicated. (B) Measurement of glucocorticoid repression of rPOMCneo mRNA transcripts by RNase mapping as described in the legend to Fig. 4A. Cellular β- and γ-actin mRNAs were measured as an internal control by Northern blots, using a rat β-actin cDNA probe.

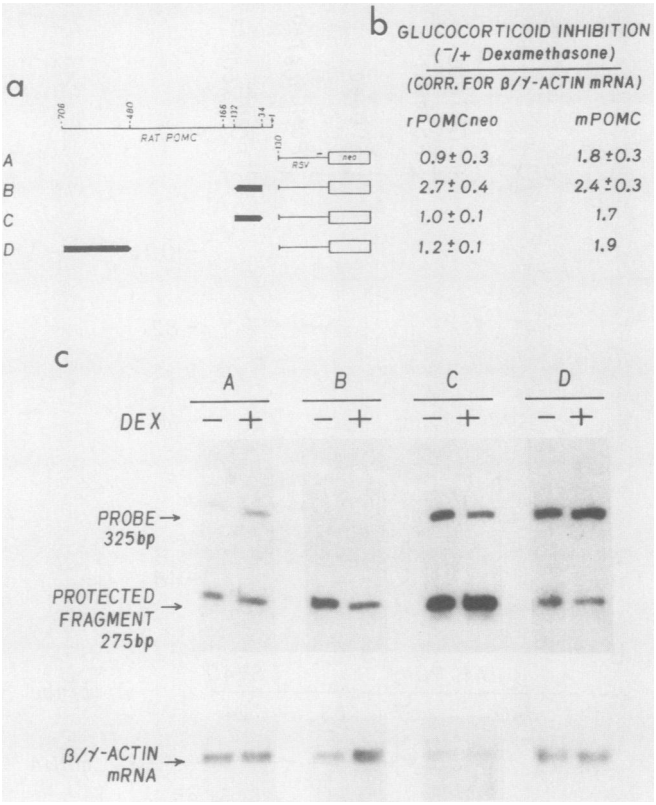

FIG. 6. Ability of POMC promoter sequences to confer glucocorticoid repression to a heterologous promoter. (A) A shortened RSV LTR promoter present in plasmid $pRSV_{130}neo$ was used to test the transcriptional properties of POMC 5'-flanking DNA fragments, which are represented in panel a as dark arrows aligned under a map of the POMC promoter. Plasmids containing units B, C, and D have 16, 21, and 1.3 times the basal promoter activity of plasmid A when electroporated in AtT-20 cells (36). The POMC fragment present in constructs B and C contains the bp −63 GR-binding site; construct D contains the bp −579 GR site. RSVneo transcripts present in AtT-20 cells electroporated with the indicated plasmids were quantitated by S1 nuclease mapping, using a 325-bp end-labeled fragment (probe) that includes 275 transcribed nt (protected fragment). Cells were treated overnight with solvent (−) or Dex (+), as indicated (c). Since RSVneo transcripts were less abundant in cells electroporated with plasmids A and D, longer exposures of the same S1 mapping autoradiographs were used. Densitometric quantitation of the protected fragment present in this and two other autoradiograms (after correction for β- and γ-actin mRNA levels) was used to calculate the magnitude of Dex-dependent glucocorticoid inhibition of rPOMCneo mRNA transcripts (b); glucocorticoid inhibition of mPOMC mRNA was similarly quantitated (b) by using Northern blots to reveal the mRNA (data not shown).

directly by insertion of a double point mutation that alters receptor binding. This mutation reduced in vitro receptor binding in the DNase I footprinting assay and abolished glucocorticoid repression (Fig. 7). The same mutation also showed reduced GR binding in a gel retardation assay, and it did not affect basal promoter activity (data not shown). The mutation involved guanosine residues that are in intimate contact with the receptor, as revealed in DMS protection experiments; these residues are located 10 bp or one DNA helix turn upstream of the hexanucleotide motif, and they are conserved in the POMC bp −63 site and in GREs. Thus, in vitro binding of the GR to the bp −63 site is closely correlated with glucocorticoid-dependent repression of POMC gene transcription, indicating that receptor binding to POMC DNA is required for repression.

DISCUSSION

Since the intracellular actions of glucocorticoids appear to be mediated by the GR, it is intriguing to speculate how this receptor can mediate both induction and inhibition of different genes. Using the POMC gene as a model, we have undertaken to study the mechanism of glucocorticoid repression of transcription. In this report, we have identified a *cis*-regulatory element, the nGRE, that contains a binding site for the GR and mediates glucocorticoid-dependent repression of POMC gene transcription.

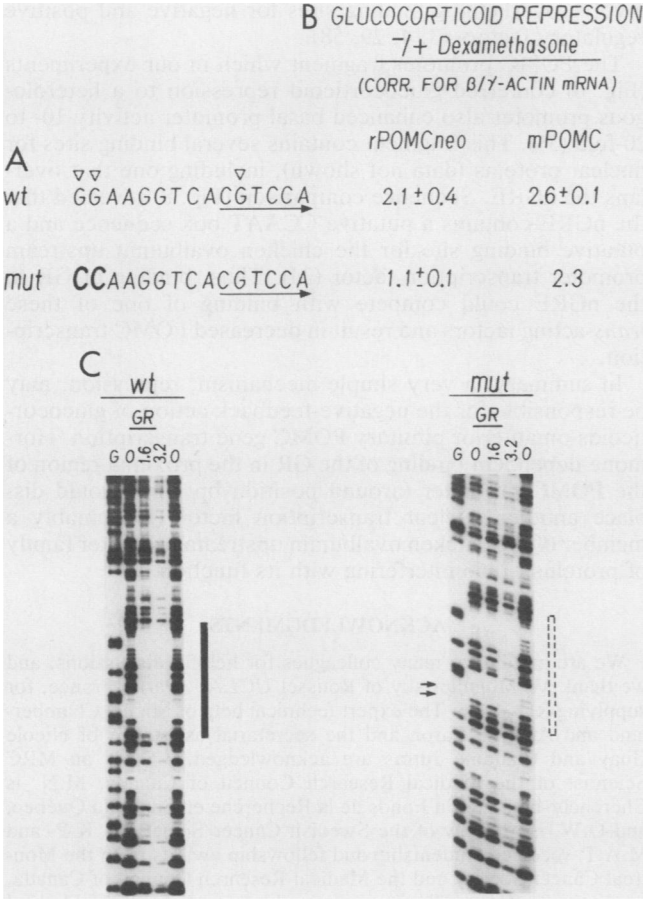

FIG. 7. Abolition of glucocorticoid repression by a point mutation that impaired GR binding. (A) A double point mutation (indicated by boldface letters) was designed to change within the nGRE a pair of guanine residues that were protected in DMS protection experiments (▽) and was introduced in the POMC promoter of plasmid $pPOMC_{480}neo$ (wt). The two plasmids were tested for in vitro binding activity by using purified rat liver GR (C) and for glucocorticoid repression after electroporation in AtT-20 cells (B). For each plasmid, GR-dependent DNase I footprints are indicated by bars, and mutated residues are indicated by arrows alongside the G sequencing lane. FPLC-purified rat liver GR was used at 1.6 and 3.2 pmol per assay, as indicated. Glucocorticoid inhibition of endogenous mPOMC and rPOMCneo mRNA transcripts was assessed in the same RNA samples by using Northern blots and, sequentially, *neo*, mPOMC, and β-actin probes. After correction for β- and γ-actin mRNA levels, which were not affected by Dex treatment, glucocorticoid inhibition was assessed by densitometry of mPOMC and rPOMCneo bands. Basal promoter activity was not significantly affected by the mutation (not shown). Data are averages and standard deviations of three independent determinations.

The POMC nGRE. We have localized an element within the rat POMC promoter that is responsible for glucocorticoid repression of transcription. This nGRE contains one of the four GR-binding sites identified in the POMC gene (Fig. 1 to 3). Deletion (Fig. 5) or site-specific mutagenesis (Fig. 7) of this GR-binding site abolished glucocorticoid sensitivity. Furthermore, a promoter fragment containing this bp −63 site conferred glucocorticoid repression to a heterologous promoter (Fig. 6). Since inclusion of the other GR-binding site present in the POMC promoter (bp −579 site) did not produce greater glucocorticoid inhibition when present together with the bp −63 site (Fig. 5) or when placed upstream of a heterologous promoter (Fig. 6), we conclude that this GR-binding site is not essential for glucocorticoid-dependent repression.

The threefold glucocorticoid-dependent repression conferred by POMC promoter fragments correlates well with the threefold decrease in endogenous mPOMC mRNA levels observed in glucocorticoid-treated AtT-20 cells (45; Fig. 4 and 5) and with the threefold decrease in POMC gene transcription rate measured after Dex treatment of rat anterior pituitary cells in primary culture (23, 24). In addition, we have recently shown in transgenic mice that the bp −706 POMC promoter fragment confers anterior pituitary-specific glucocorticoid regulation of the same magnitude as that of the endogenous POMC gene (63), which suggests that repression of POMC gene transcription by glucocorticoids is due only to transcriptional regulation and does not involve any posttranscriptional events.

The nGRE defined in this work has properties analogous to those of glucocorticoid-inducible GREs insofar as nGRE activity is dependent on receptor-DNA interactions and can be conferred to a heterologous promoter. However, the activity of the POMC nGRE appears more dependent on its position within a promoter, since many POMC DNA fragments containing the nGRE did not confer repression to heterologous promoter constructs (Fig. 6 and data not shown). This may also be the reason why synthetic oligonucleotides containing the nGRE (either 25 or 35 bp) did not confer glucocorticoid repression (or induction) when inserted upstream of the thymidine kinase or RSV promoter (data not shown). In contrast, GRE oligonucleotides were shown to confer hormone responsiveness in similar conditions (62). These observations suggest that nGRE activity may require interactions of the nGRE with other elements and their cognate *trans*-acting factors; the activity may also depend on a specific localization within the promoter.

GR binding to the POMC nGRE. Our in vitro binding studies showed that the GR binds with similar (or slightly lower) affinity to sites in the POMC and MTV genes (Fig. 2). The POMC bp −63 receptor-binding site is the only one required for repression. It is interesting to speculate whether receptor binding to this site differs from binding to GREs, which would account for their opposite transcriptional properties.

Putative differences in receptor-DNA interactions may result from differences in the primary DNA sequence of the binding sites. The sequences of the POMC and the major MTV receptor-binding sites (bp −176) are compared in Fig. 8. The DMS protection pattern of guanine residues within the POMC bp −63 site is similar to the pattern observed in the MTV receptor-binding sites (59). This finding suggests that receptor-DNA recognition takes place in a similar fashion in the major groove of the double helix. However, the intimate interaction between receptor and DNA may differ as a result of nucleotide differences within the recognition sequence. In this context, it is noteworthy that the hexanucleotide 5′-TGTYCT-3′ is invariably present in all functional GREs (5). In contrast, the DNA sequence of the bp −63 site varies significantly within its hexanucleotide motif, 5′-CGTCCA-3′. Only two receptor-binding sites containing a divergent base at the last position of the hexanucleotide were observed in glucocorticoid-inducible genes, and they were shown to be inactive (42, 54). In addition to these differences, the POMC bp −63 site does not contain the conserved ACA sequence that is located 6 bp upstream of the hexanucleotide and forms part of a dyad symmetry in GREs (62). Interestingly, in the POMC bp −63 binding site,

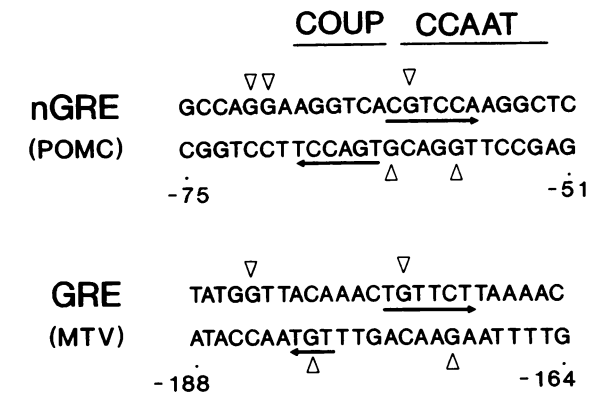

FIG. 8. Comparison of GR-binding sites present in the POMC nGRE and in the GRE of MTV. The nucleotide sequences shown are those protected by receptor in DNase I footprinting experiments. Guanine residues that were hypomethylated by DMS in the presence of receptor are indicated (▽). Arrows indicate hexanucleotides that are homologous to the consensus 5′-TGTYCT-3′ present in most GR-binding sites. A putative CCAAT box sequence and a sequence homologous to a consensus sequence binding the chicken ovalbumin upstream promoter (COUP) transcription factor (34) are shown.

two hexanucleotide sequences are present on opposite strands; however, these symmetrical motifs are juxtaposed in POMC, whereas they are separated by 3 bp in GREs. In this regard, it is interesting to note that the spacing between palindromic sequences of an estrogen response element appears to be important for the activity of that element (27).

It is not inconceivable that sequence differences between the nGRE and GREs lead to different interactions of these sequences with the same receptors. In yeast cells, for example, binding of the HAP1 activator protein to the upstream activation sequences of the *CYC1* and *CYC7* genes involves different protein-DNA interactions; the DNA sequences of these two binding sites are different, and mutations in *HAP1* revealed that different domains of the protein selectively affected binding to one site (50). In the case of the GR, protein domains required for DNA binding and transcriptional activation were identified (26, 28, 32), but the recent analysis of GR mutants affecting activation and repression of transcription revealed different requirements for the two activities (47). In addition, recent results provided evidence for two steps in hormone-dependent activation of steroid receptors (65); thus, differences between receptor-GRE and receptor-nGRE interactions could result in transcription activation and repression, respectively.

Mechanism of GR-dependent repression. While differences in receptor-DNA interactions could contribute to opposite transcriptional activities, glucocorticoid repression of transcription may also result from receptor binding in an essential region of the promoter and interference with the activity of other transcription factors; thus, the GR may behave as a hormone-dependent repressor. This mode of action is compatible with our data, regardless of the nature of the receptor-DNA interaction. In procaryotes, repressors recognize DNA sequences that overlap the promoter and thus compete directly to prevent RNA polymerase binding (55). Similarly, in yeast cells the activity of negative regulatory elements is dependent on their position between proximal and distal elements of the promoter (8), which suggests that binding at these regulatory sites disrupts essential protein-protein interactions (37). The few cases of negative control of transcription in eucaryotes that have been studied also appear to involve overlapping binding sites for negative and positive regulatory factors (3, 4, 29, 58).

The POMC promoter fragment which in our experiments (Fig. 6) conferred glucocorticoid repression to a heterologous promoter also enhanced basal promoter activity 10- to 20-fold (36). This fragment contains several binding sites for nuclear proteins (data not shown), including one that overlaps the nGRE. Sequence comparison (Fig. 8) revealed that the nGRE contains a putative CCAAT box sequence and a putative binding site for the chicken ovalbumin upstream promoter transcription factor (34). Thus, binding of GR to the nGRE could compete with binding of one of these *trans*-acting factors and result in decreased POMC transcription.

In summary, a very simple mechanism, repression, may be responsible for the negative-feedback action of glucocorticoids on anterior pituitary POMC gene transcription. Hormone-dependent binding of the GR in the proximal region of the POMC promoter (around position bp −63) would displace another nuclear transcription factor (presumably a member of the chicken ovalbumin upstream promoter family of proteins), thus interfering with its function.

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