# Structure/Activity Relationships for GMEB-2: The Second Member of the Glucocorticoid Modulatory Element-Binding Complex

Jun Chen,‡ Yuanzheng He, and S. Stoney Simons, Jr.*

*Steroid Hormones Section, NIDDK/LMCB, National Institutes of Health, U.S. Department of Health and Human Services, Bethesda, Maryland*

*Received July 29, 2003; Revised Manuscript Received October 28, 2003*

ABSTRACT: The position of the dose−response curve of agonist complexes of glucocorticoid receptors (GRs), and the partial agonist activity of GR-antagonist complexes, can be modulated by two proteins (GMEB-1 and -2), which bind as oligomers to a DNA element that is called a glucocorticoid modulatory element, or GME. This element is active when located upstream of the glucocorticoid response element that controls the expression of a reporter gene. Here, we report the structure/activity relationships of GMEB-2 and compare them to our previous findings for GMEB-1. Most of the activities of GMEB-2, such as homo- and heterooligomerization, binding to GR and to CBP, DNA binding, and modulation of the above GR transcriptional properties, require large regions of the protein. Only the intrinsic transactivation activity could be localized to a small region of the protein. These studies shed light on the mechanism of action of GMEB-2 and further support our previous conclusion that the ability of factors to modulate the position of the dose−response curve, and the partial agonist activity, of GR complexes is unrelated to effects on the total levels of GR-induced gene expression. These studies also identify regions of GMEB-2 possessing yet unidentified properties that are critical for several activities. Finally, as the domain organization of GMEB-2 and -1 is extremely similar, we conclude that the quantitative differences in activities derive from variations in amino acid sequence rather than more global features of protein structure.

A major function of steroid receptors is to convert the information from circulating concentrations of steroid hormones into different levels of specific proteins by regulating the transcriptional activities of the appropriate genes. The general mechanism by which this is achieved is similar for all of the steroid receptors and involves steroid binding to the cognate intracellular receptor, activation to a form with increased affinity for DNA, and binding to specific biologically active DNA sequences called hormone response elements (HREs) that are usually in the promoter region of genes. These DNA-bound, receptor−steroid complexes then regulate the rates of transcription from nearby DNA sequences encoding various proteins via still poorly understood mechanisms. An ever increasing number of transcriptional cofactors have been found to assist/mediate the transcriptional control signals that are initiated by steroid receptors (*1*−*4*). These cofactors were initially discovered on the basis of their ability to increase, or decrease, the total levels of gene transcripts produced by saturating concentrations of ligand. More recently, it has become clear that these same factors, in addition to several others, additionally affect two other important properties of steroid receptors: the position of the dose−response curve of receptor−agonist complexes and the partial agonist activity of receptor−antagonist complexes (*5*−*12*).

Agonists are ligands that mimic the activity of endogenous steroid hormones. The dose−response curve of agonists gives the amount of gene induction by any concentration of steroid, with half of the maximal induction occurring at a value called the $EC_{50}$. The lower the $EC_{50}$ of a given gene, the greater the level of induction that is achieved with the circulating concentration of steroid in a cell or animal. If different genes have different $EC_{50}$ values in response to the same receptor−steroid complex, then there will be a differential control of gene expression by the single concentration of endogenous steroid. Antisteroids, or antagonists, block the action of agonist steroids. However, virtually all antisteroids display some partial agonist activity with selected genes. Recently, it has been appreciated that if one can selectively eliminate the transactivation of a target gene with a specific antagonist while retaining the expression of most of the other genes that are regulated by a given receptor, then the number of undesirable side effects that usually result from the indiscriminate repression of all responsive genes by antisteroids will be greatly reduced. Thus, the ability of various cofactors to modulate the dose−response curves of receptor−agonists and the partial agonist activity of receptor−antagonist may be of major importance for differential control of gene expression during development, differentiation, and homeostasis and for endocrine therapies of a variety of conditions such as pregnancy (*13*), inflammation (*14*), and hormone-dependent cancers (*15*, *16*).

The first factor to be described to modulate the dose−response curve, and partial agonist activity, of a receptor−steroid complex was a cis-acting element from the rat

* Address correspondence to the following author. Phone: (301) 496-6796. Fax: (301) 402-3572.

‡ Current address: Pharmacology Department, C2040, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814.

10.1021/bi035311b 
Published on Web 12/09/2003

tyrosine aminotransferase gene, which was called a glucocorticoid modulatory element (GME) (*17*). The activity of the GME correlates with the binding of a species (*17*) that was subsequently found to be a complex of about 550 kDa consisting of two proteins, called glucocorticoid modulatory element binder (GMEB)-1 and -2 (*18*). GMEB-1 and -2 are 39% homologous and share a 93 amino acid sequence that is 80% identical (*19*, *20*), even though the two genes encoding the proteins reside on different chromosomes (*21*).

The GMEBs are members of an emerging new family of transcription factors that share an approximately 80 amino acid sequence, which is called a SAND domain (Sp100, AIRE-1, NucP41/75, DEAF-1) (*19*, *20*, *22*, *23*). The GMEBs have intrinsic transactivation activity and can modify the induction properties of glucocorticoid receptor (GR)−agonist and −antagonist complexes (*19*, *20*, *24*). Other properties of the GMEBs include homo- and heterooligomerization, binding to the GME, complexation with GR, interaction with CBP, and association with Ubc9 (*18*−*20*, *24*, *25*). The GMEBs have been described to interact with additional proteins, although most of the biological consequences are yet to be defined. Thus, GMEB-1 binds to hsp27 (*26*), which is an antiapoptotic protein that acts, in part, by delaying the release of cytoplasmic cytochrome *c* (*27*) and that increases the tumorigenic potential of rat colon carcinoma cells (*28*). GMEB-1 also associates with the second activation domain (AD2) of the human coactivator TIF2 (29) in yeast in a manner that may be mediated by an adapter molecule (*30*) and binds to MURF-1 (muscle-specific RING finger-1) (*31*), which binds to the C-terminal region of the giant sarcomeric protein titin. GMEB-1 has recently been reported to be the same as the human STAT6 activating gene (*32*). Most interesting are the observations that the GMEBs are involved in parvovirus replication, where they are called PIFs (parvovirus initiation factors) (*33*, *34*) and activate the viral nickase NS1 (*35*). While the heterocomplex of GMEBs/PIFs is able to activate the nicking activity of NS1, the homooligomer of GMEB-2, but not of GMEB-1, is also able to activate nicking (*35*).

To understand the molecular mechanisms of GMEB action, and the different roles of the GMEBs in modulating GR induction properties versus replicating parvoviruses, it is necessary to identify the sequences that mediate each biological response. The structure−activity relationships of GMEB-1 have been reported (*24*). The purpose of this study is to identify the domains of the various activities of GMEB-2 that are relevant for modulating GR properties and to determine the extent to which GMEB-1 and -2 share similar domain organization and structures. We have now characterized the new DNA-binding motif for these members of the SAND-domain family of proteins. We find that the organization of functional domains is remarkably similar for GMEB-2 and -1 despite their sequence differences. Furthermore, with both GMEBs, multiple domains are required for the expression of several of the biological activities. This information helps to explain how the GMEBs work and will be useful in identifying those proteins that are required to bind to the GMEBs to give the eventual biological responses.

## MATERIALS AND METHODS

Unless otherwise indicated, all operations were performed at 37 °C.

*Chemicals*. The following chemicals were purchased from the indicated sources: [$^{35}$S]Met, Amersham Pharmacia Biotech (Piscataway, NJ); dexamethasone (Dex), Sigma (St. Louis, MO); prestained molecular weight markers, Lipofectamine plus, Herring Sperm DNA, and oligonucleotides, Life Technologies, Inc. (Gaithersburg, MD); acrylamide and bisacrylamide, National Diagnostics (Atlanta, GA); TNT-coupled reticulocyte lysate system, Promega (Madison, WI); cross-linking reagent (EGS), Pierce (Rockford, IL); and restriction enzymes and DNA polymerase, New England Biolabs (Beverly, MA), Life Technologies, Inc., and Promega.

*Plasmids*. The Renilla-TS reporter was a gift from Drs. Nasreldin M. Ibrahim and Otto Fröhlich (Department of Physiology) and Dr. S. Russ Price (Department of Medicine) at Emory University School of Medicine (Atlanta, GA).

With regard to the various constructs of GMEB-2, a shorthand nomenclature was used where B2 = GMEB2, N = the N-terminal amino acid, C = the C-terminal amino acid, and the remaining sequences after deletions are indicated by the numbers in parentheses of the amino acid positions, using N for position 1 and C for position 530. Therefore, B2(N347) corresponds to amino acids 1−347 of GMEB-2.

*HisB2 (His/GMEB-2) Constructs*. HisB2: the NcoI and HindIII double digest of GMEB2/pCRII (*19*) was filled in with Klenow and inserted into the EcoRV site of pcDNA3.1HisA (Invitrogene). HisB2(GD45IS): changed GGGTGAT to GatatcT using the site-specific mutagenesis kit, GeneEditor (Promega). HB2(47C): the EcoRV and XbaI double digest of HisB2(GD45IS) was inserted into EcoRV and XbaI digested pcDNA3.1HisA. HisB2(DG96IS): changed GTGGAGAC to GgatAtcC using site-specific mutagenesis (GeneEditor from Promega). HB2(99C): the EcoRV and XbaI double digest of HisB2(DG96IS) was inserted into EcoRV/XbaI digested pcDNA3.1HisA. HisB2(125C): the BclI/HindIII cleaved HisGMEB2 was inserted into BamHI/HindIII cut pcDNA3.1HisA. HisB2(185C): Bsp120I/HindIII digested HisGMEB2 was inserted into HindIII/NotI cleaved pcDNA3.1HisA. HisB2(309C): XhoI fragment of HB2 was deleted and religated. HisB2(423C): Crf10/HindIII treated HisGMEB2 was inserted into EcoRV/HindIII cut pcDNA3.1HisA. HisB2(K139I/D140S): changed GAAGGAC to GAtatcC using site specific mutagenesis (GeneEditor from Promega). HisB2(N125): BcII/XbaI digested HisGMEB2 was inserted into EcoRV/XbaI treated pcDNA3.1HisA. HisB2(N185): Bsp120I /XbaI cut HisGMEB2 was inserted into BamHI/XbaI cleaved pcDNA3.1HisA. HisB2(N309): XhoI fragment of pmB2 was deleted and religated. HisB2(N347): HincII/HindIII treated HisGMEB2 was inserted into EcoRV/HindIII digested pcDNAHisA. HisB2(N369): BsmBI/PvuII cut HisGMEB2(N423) was inserted into BsmBI/XbaI cleaved HisGMEB2(N423). HisB2(N423): Cfr10/XbaI treated HisGMEB2 was inserted into EcoRV/XbaI digested pcDNA3.1HisA.

*pmB2 (GAL/GMEB-2) Constructs.* pmB2: BamHI cleaved HisGMEB2 was inserted into BamHI digested pm (from Clontech). pmB2(125C): BclI/XbaI treated HisGMEB2 was inserted into BamHI/XbaI cleaved pm. pmB2(185−309): cut pmB2(N309) with SmaI/Bsp120I and self-religated. pmB2(185C): BamHI treated HisGMEB2(185C) was inserted into BamHI digested pm. pmB2(214−309): BspMI/

XbaI treated pmB2(N309) was inserted into BamHI/XbaI cut pmB2(N309). pmB2(309C): BamHI digested HisGMEB2-(309C) was inserted into BamHI treated pm. pmB2(47C): EcoRV/XbaI cut HisB2(GD45IS) was inserted into EcoRV/XbaI digested pmB2(N125). pmB2(N125): BamHI/XbaI cleaved HisGMEB2(N125) was inserted into BamHI/XbaI treated pm. pmB2(N185): BamHI/XbaI cut HisGMEB2-(N185) was inserted into BamHI/XbaI treated pm. pmB2(N263): BamHI/StuI digested pmB2(N309) was inserted into EcoRV/BamHI cut pmB2(N125). pmB2(N299): MscI/XbaI treated pmB2(N309) was deleted, filled-in, and religated. pmB2(N309): BamHI/XbaI digested HisGMEB2(N309) was inserted into BamHI/XbaI treated pm. pmB2(N58): EcoRV/PvuII cleaved pmB2(N125) was deleted, filled-in, and religated.

*pVP16-B2 (VP16/GMEB-2) Constructs*. pVP16-B2: EcoRI/XbaI cut pmB1(307C) was inserted into EcoRI/XbaI treated pVP16 (Clonetech). pVP16-B2(185C): BspEI/BamHI generated N-terminal fragment of VP16B2 was replaced with the BspEI/BamHI digest of pmB2(185C). pVP16-B2(214−309): EcoRI/SapI treated pmB2(214−309) was inserted into EcoRI/SapI cut pVP16. pVP16-B2(309C): EcoRI/PflMI N-terminal fragment of VP16B2 was replaced with EcoRI/PflMI digested pmB2(309C). pVP16-B2(47C): BamHI treated HisB2(47C) was inserted into BamHI cleaved VP16B2. pVP16-B2(N125): BamHI/XbaI cut pmB2(N125) was inserted into BamHI/XbaI digested pVP16. pVP16-B2(N185): BamHI/XbaI treated pmB2(N185) was inserted into BamHI/XbaI cut pVP16. pVP16-B2(N263): AccI/SapI cleaved pmB2(N263) was inserted into AccI/SapI treated pVP16. pVP16-B2(N299): BamHI/SapI cut pmB2(N299) was inserted into BamHI/SapI cut pVP16. pVP16-B2(N309): BamHI/XbaI treated pmB2(N309) was inserted into BamHI/XbaI cleaved pVP16.

*GEX-4T-GMEB2 Constructs*. GEX-4T-GMEB2: EcoRI digested pcDNA3.1HisGMEB2 was inserted into EcoRI cut GEX-4T-2. GEX-4T-GMEB2(185C): EcoRI treated pcDNA3.1HisAGMEB2(185C) was inserted into EcoRI treated GEX-4T-2. GEX-4T-GMEB2(309C): EcoRI cleaved pcDNA3.1HisAGMEB2(309C) was inserted into EcoRI cut GEX-4T-2. GEX-4T-GMEB2(47C): EcoRI treated pcDNA3.1HisAGMEB2(47C) was inserted into EcoRI cut GEX-4T-2. GEX-4T-GMEB2(N185): EcoRI/HincII digested pmGMEB2(N185) was inserted into EcoRI/SmaI treated GEX-4T-2. GEX-4T-GMEB2(N263): the large fragment of GEX-4T-GMEB2 after StuI/SmaI cleavage was self-ligated.

*In Vitro Expression of Proteins*. All cDNAs of the protein to be expressed were cloned so that they were under the control of either the T7 or the SP6 promoter. For each reaction, 1 $\mu$g of plasmid DNA was mixed with 2 $\mu$L of 1 mM methionine and 40 $\mu$L of TNT T7 (or SP6) master mix (Promega) and brought up to a total volume of 50 $\mu$L with $H_2O$. The reaction is conducted at 30 °C for 2 h. For radiolabeling the protein, 2 $\mu$L of 1 mM methionine was replaced with 2 $\mu$L of [$^{35}$S]methionine (Amersham Pharmacia Biotech). When in vitro translating GR, 1 $\mu$L of 50 $\mu$M Dex is added in the reaction mix to bind, and thereby stabilize, the newly translated GR.

*Bacterial Expression of Proteins*. GST/GMEB-2 constructs were transformed into BL21 bacteria (Pharmacia) according to the manufacturer's procedure. A single colony was selected, inoculated into 20 mL of LB broth with 100 mM ampicillin, and cultivated overnight at 37 °C in a shaking incubator. A portion (15 mL) of the overnight culture was inoculated into 150 mL of LB broth (with 100 $\mu$g/mL Ampicillin), shaken at 37 °C for 3−4 h, and then induced with 0.1 or 2−3 mM isopropyl $\beta$-D-thiogalactopyranoside for 3−4 h. Cells were washed once with Dulbecco's phosphate buffered saline (PBS; GIBCO), resuspended in 10 mL of PBS, and sonicated for 30 cycles (1 cycle = 10 s on and 10 s off, Braunsonic Model 1510). Cell lysates were obtained by spinning the sonicated cells at 20 800*g* for 15 min and collecting the supernatant.

*Pulldown Assay*. Glutathione-Sepharose 4B beads (30 $\mu$L; Amersham Pharmacia Biotech) were added to each tube and washed twice with 500 $\mu$L of PBS (6000*g* for 2 min). Bacterial lysates (500−1500 $\mu$L) containing glutathione-*S*-transferase (GST) ± fused heterologous protein were mixed gently with the beads by rotation (12−15 rpm) for 1−2 h at 4 °C. The pellets were isolated by centrifugation (2 min at 6000*g*) with two washes of 500 $\mu$L of PBS containing 10 mM $\beta$-mercaptoethanol. Dex-bound $^{35}$S-labeled GR (7−10 $\mu$L) was added and incubated at 4 °C overnight with rotation. The beads were washed twice with 500 $\mu$L of PBS (+10 mM $\beta$-mercaptoethanol) and then five times with PBS (+100 mM NaCl). Proteins were removed from the beads by heating at 90 °C for 10−15 min in 40 $\mu$L of 2× sample buffer (Quality Biological, Inc., Gaithersburg, MD). Aliquots (10−15 $\mu$L) of the supernatant were loaded onto 10% SDS−PAGE mini-gels (200 V for 45 min). One gel was dried for 25 min and then used to expose film or a phosphorimaging screen. The other gel was analyzed by Western blotting with anti-GST antibody to detect the GST fusion proteins.

*Western Blotting*. SDS−PAGE gels were equilibrated in transfer buffer for 15 min at room temperature prior to the electrophoretic transfer of proteins to nitrocellulose membranes in a Bio-Rad small (150−200 mA overnight) or large (350 mA overnight) transblot apparatus. The nitrocellulose was stained in Ponceau S (0.02% Ponceau S and 0.04% glacial acetic acid in water) to localize the molecular weight markers, incubated with 5% Carnation nonfat dry milk in Tris buffered saline (TBS; Quality Biological, Inc.) for 30−45 min, and washed three times with TBS containing 0.2% Tween (0.1TTBS) for 5 min. Primary antibody was diluted in 0.2TTBS (1:10 000 for anti-VP16, 1:10 000 for anti-Gal, 1:10 000 for X-press, and 1:5000 for anti-GST) and added to the nitrocellulose filters for 1−2 h at room temperature. Biotinylated anti-rabbit secondary antibody and ABC reagents (each is diluted 1:5000 except for the anti-goat antibody, which is diluted 1:50 000; Vector Laboratories, Burlingame, CA) were each added for sequential 30 min incubations at room temperature. After the incubation periods with primary antibody, secondary antibody, and ABC reagents, the nitrocellulose was washed four times for 5 min each with 0.1TTBS. The signals were detected by enhanced chemiluminesence using the recommended protocol of the supplier (Amersham).

*Cross-Linking Assay*. In vitro translated protein (5 $\mu$L) in 40 $\mu$L of water, or PBS, at 0 °C was treated with 5 $\mu$L of DMSO containing 0, 10, or 100 mM cross-linking reagent (EGS; PIERCE). After brief vortexing (2−5 s), the mixture was incubated for 15−30 min before adding 10 $\mu$L of 1 M Tris-HCl to stop the reaction. Samples (10 $\mu$L) were then assayed on 10% SDS−PAGE gels as described above.

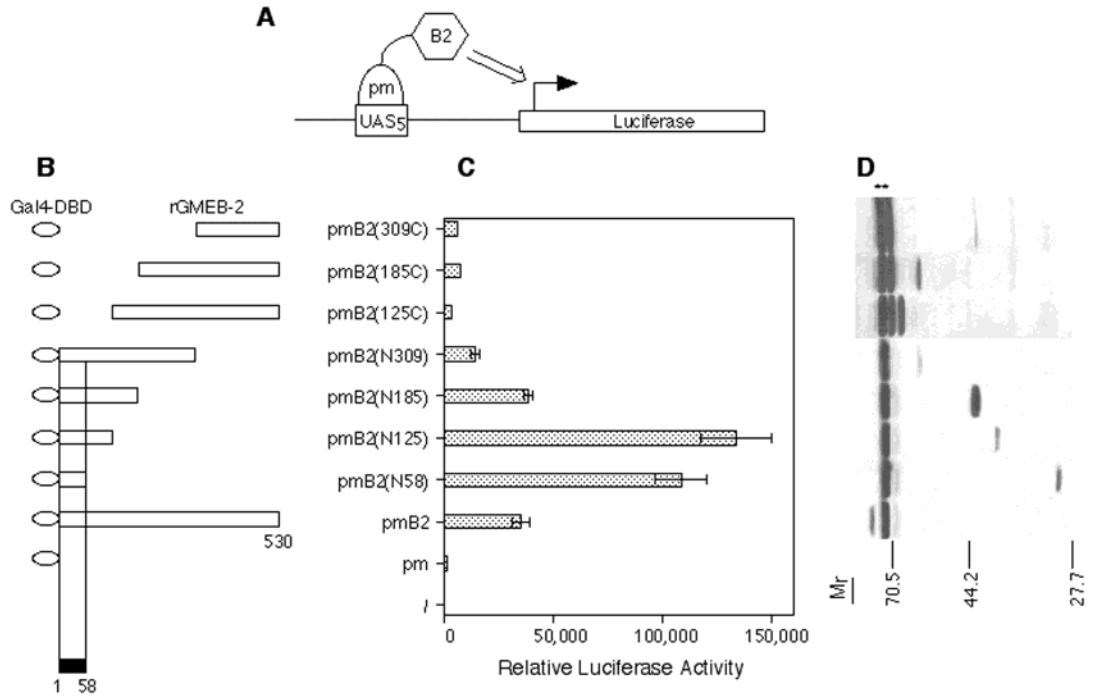


FIGURE 1: Intrinsic transactivation activity of GMEB-2. (A) Cartoon depicting the one-hybrid assay system used to evaluate the intrinsic transactivation activity of GMEB-2 sequences (pm = DNA-binding domain of GAL4 or GAL4-DBD; $UAS_5$ = five tandem repeats of the upstream activating sequence that binds GAL4-DBD). (B) Structures of the GAL/GMEB-2 chimeras used in the one-hybrid assay. (C) Biological activities of GAL/GMEB-2 chimeras in the one-hybrid assay. Duplicate samples of COS-7 cells were transiently transfected with 1 $\mu$g of the plasmid encoding the indicated GAL/GMEB-2 chimeras and 1 $\mu$g of reporter plasmid (GAL4-E1B-LUC). The induced Luciferase values were determined 20 h after transfection as described in Materials and Methods and normalized for total protein. Similar results to panel C were obtained in three additional experiments. (D) Western blots of overexpressed GAL/GMEB-2 chimeras. GAL/GMEB-2 (pmGMEB-2) chimeras were detected with anti-GAL antibody after transient transfection in COS-7 cells. The positions of molecular weight markers [kDa] are indicated on the left. Asterisks (*) indicate nonspecifically detected bands.

*Transient Transfection Assays*. COS-7 and CV-1 cells were grown on 60 mm dishes in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal calf serum. Cells were seeded 1 day before transfection at a density of $2 \times 10^5$/plate for CV-1 cells and $2 \times 10^6$/plate for COS-7 cells. Cells were transfected, using 5 $\mu$L of Lipofectamine Plus reagent and 8 $\mu$L of Lipofectamine, with 1 $\mu$g of reporter plasmid, 10 ng of RenillaTS, plus other plasmids as indicated and adjusted to a total of 3 $\mu$g per plate with herring sperm DNA. After incubating the cells at 37 °C for 5 h, the transfection mixture was replaced with normal medium. The cells were incubated at 37 °C overnight before being induced with the appropriate steroid for 24 h. The cells were lysed and assayed for reporter gene activity using the Luciferase Assay Reagent according to the manufacturer's instructions (Promega). Luciferase activity was measured in an EG&G Berthhold luminometer (Microlumat LB 96 P).

*Gel Shift Assay*. The GME-containing oligonucleotides 5′-CTTCTGTATGAGCGCCAGTAT-3′ and 3′-GAAGACAT-ACTCGCGGTCATA-5′ were annealed and $^{32}$P-end-labeled by Lofstrand Laboratories (Gaithersburg, MD). Gel shift experiments were performed as described (*19*) with minor modifications. Briefly, the in vitro transcription/translation product (1 $\mu$L) was incubated with 20 000 cpm of the $^{32}$P -end-labeled GME (0.5 fmol) in a total volume of 20 $\mu$L for 15 min with sheared, nondenatured herring sperm DNA (0.3 $\mu$g) as a nonspecific competitor. After electrophoresis in a 5% nondenaturing polyacrylamide gel at 150 V in 0.4× Tris/Borate/EDTA electrophoresis buffer (TBE), the dried gels were autoradiographed for 12−24 h at room temperature with Kodak X-Omat XAR-5 film. Alternatively, the gels were exposed to the phosphorimaging screen for the Molecular Dynamics Image-Quant system for 16−48 h at room temperature. The amount of each specific band was calculated as the intensity of that band (calculated by the Molecular Dynamics software) minus the constant background value of the same area from an unrelated region of the gel.

*Statistical Analysis*. Unless otherwise noted, all experiments were performed several times. The values of *n* independent experiments were analyzed for statistical significance by the two-tailed Student's *t* test using the program InStat 2.03 for Macintosh (GraphPad Software, San Diego, CA). When the difference between the standard deviations of two populations is significantly different, then the Mann−Whitney test or the Alternate Welch *t* test was used.

## RESULTS

*GMEB-2 Transactivation Domain*. The presence of intrinsic transactivation activity in GMEB-2 was previously demonstrated in mammalian cells when a chimera of the GAL4 DNA-binding domain (DBD) and full-length GMEB-2 was found to transactivate a Luciferase reporter containing five upstream repeats of the GAL4 response element (*20*, *25*). The same mammalian one-hybrid assay (Figure 1A) is used here with a variety of GAL/GMEB-2 (pmB2) chimeras containing different deletions in GMEB-2 (Figure 1B). The numbers in parentheses for each construct indicate the remaining GMEB-2 sequence with N and C designating the

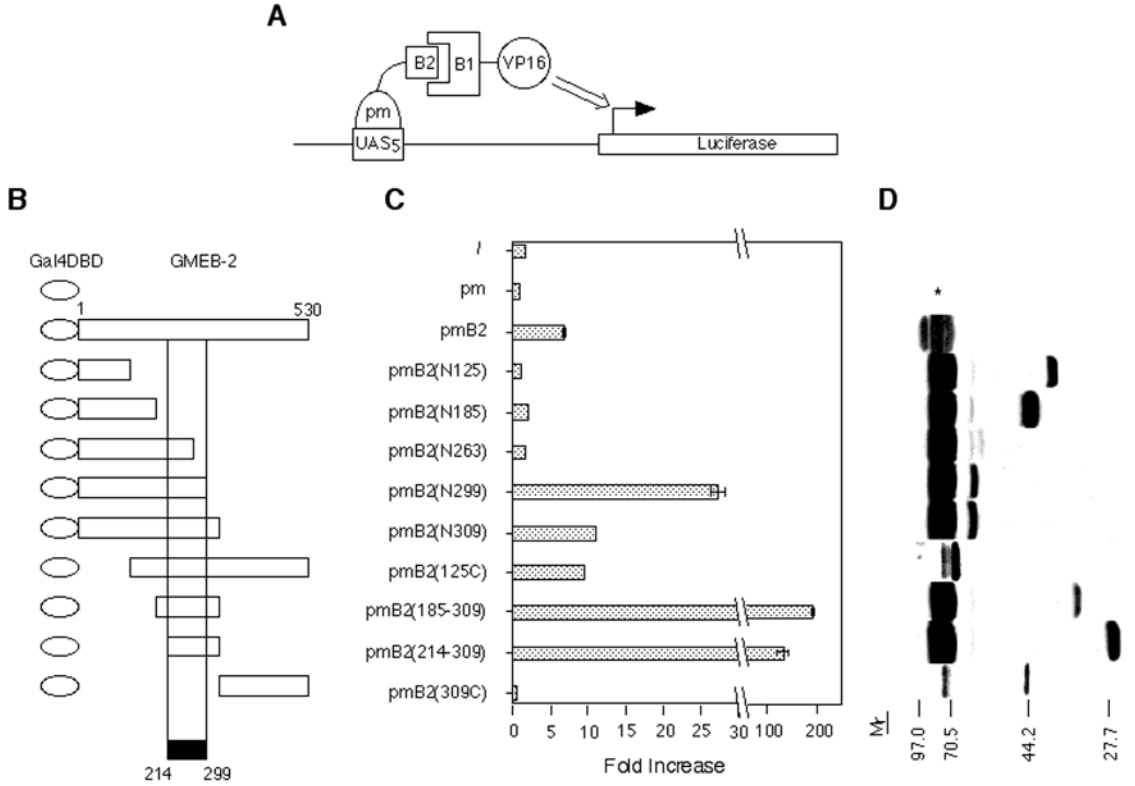


FIGURE 2: Heterooligomerization domain of GMEB-2. (A) Cartoon depicting the two-hybrid assay system used to evaluate the interaction between regions of GMEB-2 and full-length GMEB-1 (pm = DNA-binding domain of GAL4 or GAL4-DBD; VP16 = activation domain of VP16; $UAS_5$ = five tandem repeats of the upstream activating sequence that binds GAL4-DBD). (B) Structure of the GAL/GMEB-2 chimeras used in the two-hybrid assay. (C) Biological activities of GAL/GMEB-2 chimeras in two-hybrid assay with VP16/GMEB-1. Duplicate samples of COS-7 cells were transiently transfected as described in the Materials and Methods with 1 $\mu$g each of the indicated GAL/GMEB-2 chimeras and reporter (GAL4-E1B-LUC), 1 $\mu$g of either VP16 or VP16/GMEB-1 chimera plasmid, and 10 ng of Renilla plasmid as an internal control. The relative Luciferase activities were normalized for Renilla expression. The fold increase in Luciferase activity caused by the presence of GMEB-1 was then determined by dividing the normalized activity of each GAL/GMEB-2 construct with VP16/GMEB-1 by the normalized activity of the same GAL/GMEB-2 construct with VP16. Similar results were obtained in more than four additional experiments. (D) Western blots of overexpressed GAL/GMEB-2 chimeras. GAL/GMEB-2 (pmGMEB-2) chimeras were detected with anti-GAL antibody after transient transfection in COS-7 cells. The positions of molecular weight markers [kDa] are indicated on the left. An asterisk (*) indicates nonspecifically detected bands.

N- and C-terminal amino acids: for example, pmB2(N309) consists of the GAL DBD fused to amino acids 1−309 of GMEB-2. The smallest region of GMEB-2 to display high levels of activity is a fragment containing the N-terminal 58 amino acids (Figure 1C). Western blots using anti-GAL antibody (Figure 1D) show that the low activity of several proteins, such as pm/B2(125C), is not due to reduced levels of protein expression. Furthermore, the lower activity of pm/B2(N185) versus pm/B2(N125) is not due to lower amounts of protein, and therefore, may result from an inhibitory activity in the sequences between amino acids 185 and 126 (Figure 1C,D).

*GMEB-2 Heterooligomerization Domain*. GMEB-1 and -2 exist in cells as a heterooligomer with an apparent molecular weight of 550 kDa (*18*). The region of GMEB-2 required for binding to GMEB-1 was determined using a mammalian two-hybrid assay (Figure 2A). We chose to fuse GMEB-2 fragments to the GAL4-DBD, as opposed to the VP16 activation domain, because the interactions between GAL/GMEB-2 and VP16/GMEB-1 are much stronger than those of the opposite orientation (i.e., GAL/GMEB-1 and VP16/GMEB-2) (*20*). The data with the constructs of Figure 2B suggest that amino acids 214−299 are sufficient for the binding of GMEB-2 to GMEB-1 (Figure 2C). The data from each experiment were normalized to the activity of VP16/GMEB-1 with just GAL (= pm), to facilitate comparisons between different experiments, and then expressed as the fold increase for VP16/GMEB-1 over that for VP16. The approximately equal levels of expressed chimeric proteins in the Western blots of Figure 2D support our conclusion that residues 214−299 are sufficient for GMEB-2 to heterodimerize with GMEB-1.

*GMEB-2 Homooligomerization Domain*. We first used chemical cross-linking with EGS to locate the region of GMEB-2 that is needed for homooligomerization. Solutions of overexpressed His-tagged GMEB-2 constructs (Figure 3A) were treated with increasing concentrations of EGS and then analyzed on SDS gels (Figure 3B). At the highest level of EGS used, the concentration of monomeric GMEB-2 decreases, and a higher molecular weight species often appears at a position that is consistent with a dimer. With this approach, amino acids 185−309 are found to be sufficient for homooligomerization. This region is somewhat larger than the heterooligomerization domain that was identified in Figure 2. To directly compare the determinants for homo- and heterooligomerization in the same assay, we prepared the VP16/GMEB-2 constructs of Figure 3D and determined their ability to interact with GAL(pm)/GMEB-1 and /GMEB-2 in the mammalian two-hybrid assay. As shown in Figure 3C,E, VP16/B2(214−309) causes a strong response in the

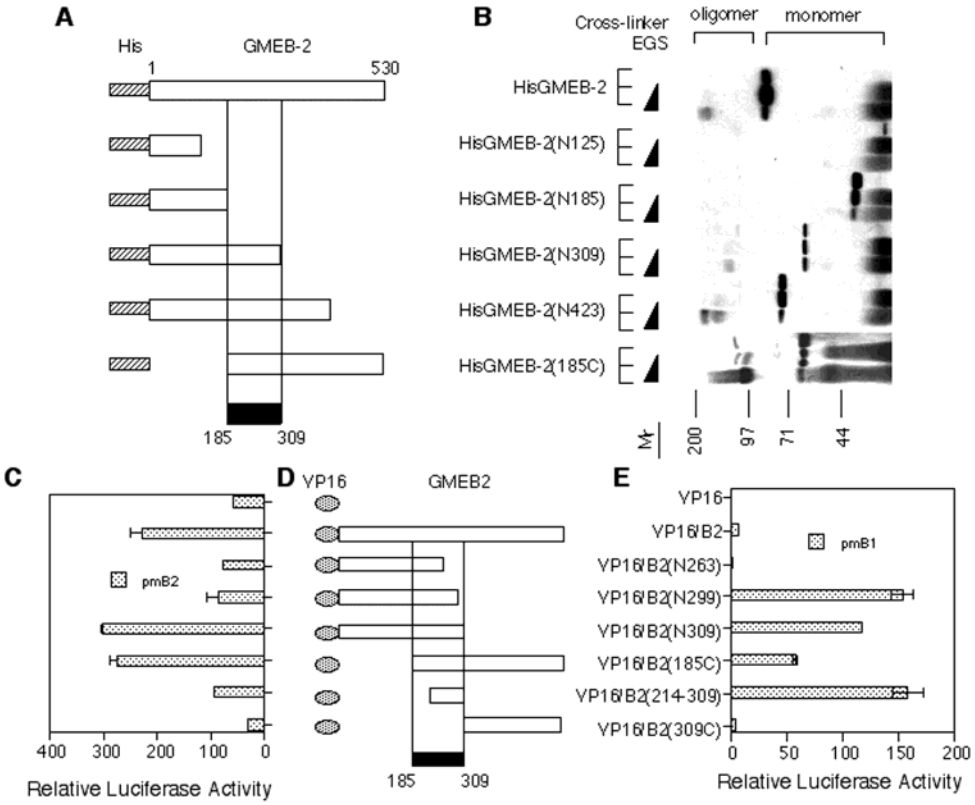


FIGURE 3: Homooligomerization domain of GMEB-2. (A) Structure of the His/GMEB-2 chimeras used in the cross-linking assay. (B) Determination of homooligomerization activity of GMEB-2 chimeras as indicated by their ability to be cross-linked. The indicated His/Xpress-tagged GMEB-2 proteins were prepared by in vitro translation. The proteins were then not treated or cross-linked with 10 or 100 mM EGS, separated by electrophoresis on 10% SDS−PAGE gels, and detected by Western blotting with anti-Xpress antibody as described in the Materials and Methods. The positions of molecular weight markers [kDa] are indicated. (C−E) Determination of homooligomerization activity of GMEB-2 chimeras in two-hybrid assays. Duplicate samples of COS-7 cells were transiently transfected as described in the Materials and Methods with 1 $\mu$g each of the indicated GAL/GMEB-2 chimeras and reporter (GAL4-E1B-LUC), 1 $\mu$g of either VP16 or VP16/GMEB-2 chimera plasmid in panel C (to determine homooligomerization activity), or VP16/GMEB-1 chimera plasmid in panel E (to confirm the activity of expressed chimera in heterooligomerizing with GMEB-1), and 10 ng of Renilla plasmid as an internal control. The relative luciferase activities were normalized for Renilla expression. Similar results to those in panel C were obtained in three additional experiments.

heterodimerization with GAL/GMEB-1 (pmB1) but is relatively inactive for homodimerizing with GAL/GMEB-2 (pmB2). Similarly, VP16/B2(N299) is active with GMEB-1 but not with GMEB-2. These data strongly support our previous conclusion that the homooligomerization domain of GMEB-2 (amino acids 185−309) is composed of the heterooligomerization domain (amino acids 214−299) plus additional residues at each end of the heterooligomerization domain.

*DNA-Binding Domain of GMEB-2*. A small portion of the SAND domain proteins that contains the amino acid sequence KDWK is thought to be required for the DNA binding of these proteins (*20*, *22*−*24*). However, there is very little direct evidence for the involvement of this sequence. To examine this in greater detail, we measured the binding of in vitro translated His-tagged GMEB-2 fragments to a GME oligonucleotide in a gel shift assay (*17*, *18*, *20*, *24*) (Figure 4). Good binding to the GME oligonucleotide requires the majority of the GMEB-2 protein sequence (Figure 4B). Interestingly, though, altering the KDWK sequence with the double point mutation of K139I/D140S in pmB2(KD140IS) eliminates the DNA-binding activity. Western blots show that this negative result is not a function of poor expression of the double mutant (Figure 4C). This result indicates that the KDWK sequence is essential for the DNA-binding activity of GMEB-2 even though many other residues are also needed.

*GMEB-2 Sequences Required for Modulation of GR Transactivation Properties*. We previously demonstrated that overexpression of the GMEBs, either individually or together, causes a right shift in the position of the dose−response curve of GR−agonist complexes and a decrease in the partial agonist activity of GR−antagonist complexes (*20*). GMEB-2 was found to be more potent than GMEB-1 in these assays. A variety of His-tagged GMEB-2 constructs were therefore prepared to identify the smallest sequence that maintained high modulatory activity in intact CV-1 cells (Figure 5A). The ability of each GMEB-2 construct to shift the dose−response curve to higher steroid concentrations was assessed by using our previously validated assay, in which a decrease in the activity of a subsaturating concentration of agonist (expressed as percent of maximal induction by saturating concentrations of agonist) is diagnostic of a right shift of the dose−response curve to higher $EC_{50}$ values (*5*, *8*, *20*, *24*). The capacity of GMEB-2 to alter the partial agonist activity of antiglucocorticoids is monitored by expressing the activity of the antagonist Dex-Mes (*36*) as percent of maximal induction by a saturating concentration of the agonist Dex. Using these assays, we find that the same region of GMEB-2 (amino acids 47−369) is required to modulate

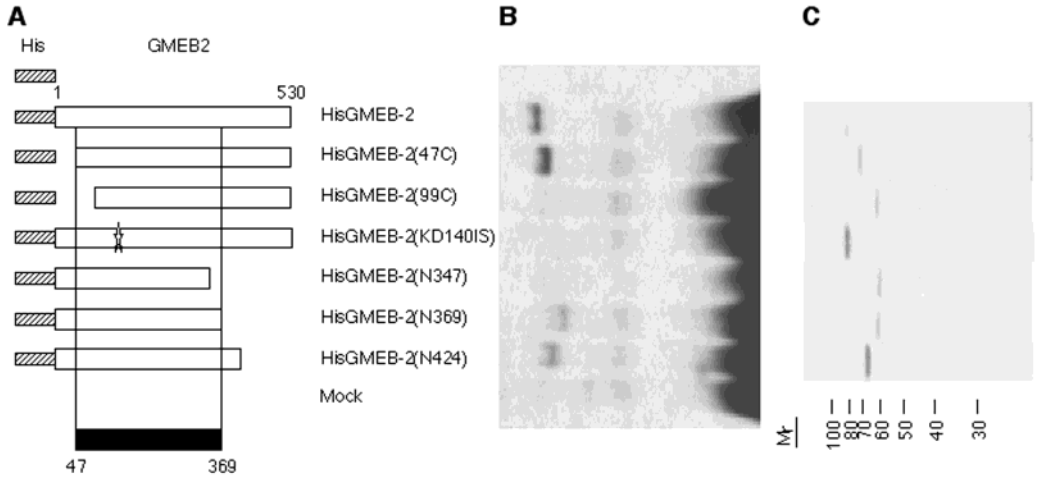


FIGURE 4: DNA-binding domain of GMEB-2. (A) Structure of the His/GMEB-2 chimeras used in the gel shift assay. The star indicates the position of the KDWK motif. (B) DNA-binding activity of GMEB-2 chimeras as assessed by their ability to bind to GME in a gel shift assay. The indicated His/Xpress-tagged GMEB-2 proteins were prepared by in vitro translation. Aliquots (1 $\mu$L) of the programmed lysate were incubated with [$^{32}$P]end-labeled GME oligonucleotide, and the resulting complexes were detected by autoradiography, as described in the Materials and Methods. Similar results were obtained for each GMEB-2 chimera in three additional experiments. (C) The expression level of the His/GMEB-2 chimeras after in vitro translation was determined by Western blotting with anti-Xpress antibody (positions of molecular weight markers [kDa] are indicated on the left).

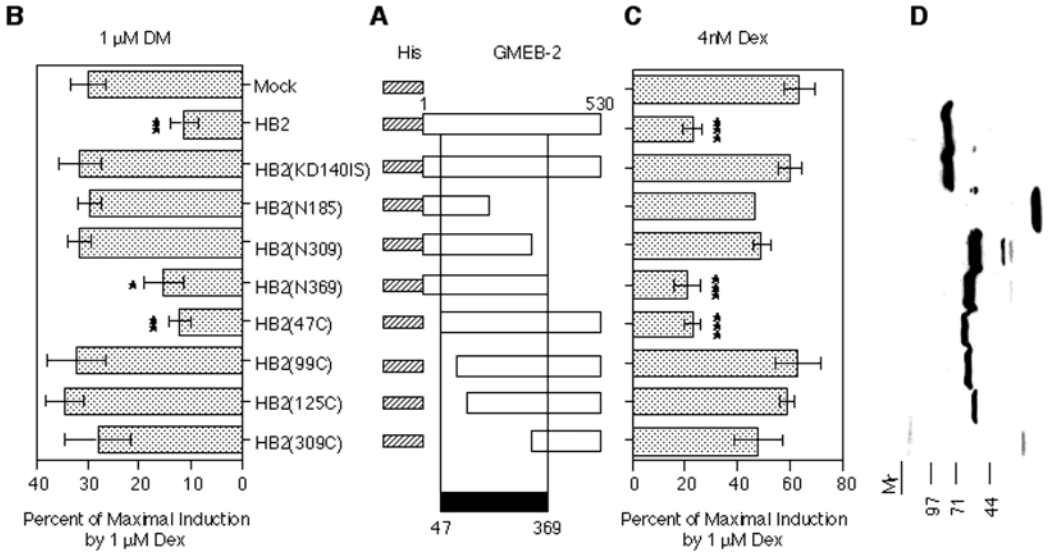


FIGURE 5: GR modulatory domain of GMEB-2. (A) Structure of the His/GMEB-2 chimeras used in the biological activity assays. (B and C) Modulatory activity of His/GMEB-2 chimeras in (B) the abbreviated dose−response curve assay and (C) the partial agonist activity assay. Triplicate samples of CV-1 cells were transiently transfected as described in the Materials and Methods with 1 $\mu$g of the GMEGREtkLUC reporter, 40 ng of GR plasmid (pSVLGR), 100 ng of His/GMEB-2 chimera (or the molar equivalent of chimera with mutant GMEB-2), and 10 ng of Renilla plasmid as an internal control. The relative luciferase activities of each chimera, after being normalized for Renilla expression, were expressed as the percent of activity seen with the His control plasmid, which lacks any GMEB-2 sequences (top lane), for (B) 1 $\mu$M Dex-Mes, or (C) 4 nM Dex. The average values from two to 10 experiments (±SEM) are plotted. The asterisks indicate those values that are significantly different from the control value at the level of $P = 0.012$ (*), $\leq 0.006$ (**), and $\leq 0.0001$ (***). (D) The expression level of the His/GMEB-2 chimeras after transient transfection in COS-7 cells was determined by Western blotting with anti-Xpress antibody (positions of molecular weight markers [kDa] are indicated on the left).

both the dose−response curve and the partial agonist activity (i.e., the percent of maximal activity with 4 nM Dex [Figure 5C] and 1 $\mu$M Dex-Mes [Figure 5B], respectively). In both cases, this amino acid sequence of 47−369 produces statistically significant reductions in activity (*P* values are * = 0.012, ** $\leq$0.006, and *** $\leq$0.0001). The Western blots of Figure 5D indicate that this conclusion is not biased by unequal amounts of expressed protein for the various GMEB-2 constructs.

*GMEB-2 Binding to GR.* A major feature of the proposed mechanism of action of the GME, and the GMEBs that bind to the GME, is that the GMEBs also interact with GR (*20*, *24*, *37*). This interaction has been documented by two-hybrid and pulldown assays (*20*, *24*). To identify the regions of GMEB-2 that contact GR, we examined the ability of GST chimeras with different regions of GMEB-2 (Figure 6A) to bind [$^{35}$S]methionine-labeled, in vitro translated GR. A region between amino acids 47 and 185 is sufficient for the binding of Dex-bound GR (Figure 6B). The Western blots of Figure 6C show that the absence of binding to the GMEB-2(185C) and (309C) chimeras is not an artifact of very low levels of protein expression.

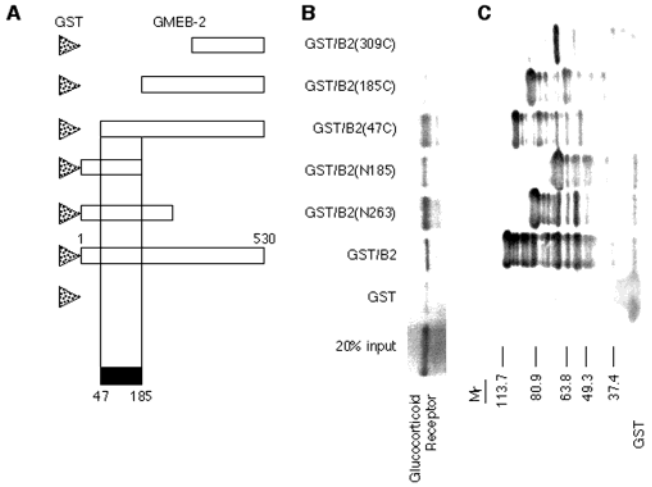


FIGURE 6: GR interaction domain of GMEB-2. (A) Structure of the GST/GMEB-2 chimeras used in the pulldown assay. (B) Immobilization of full-length GR by GMEB-2 fragments in a pulldown assay. Bacterially expressed GST/GMEB-2 chimeras, which had been immobilized on glutathione-Sepharose beads, were incubated with in vitro translated, [$^{35}$S]methionine-labeled, Dex-bound complexes of GR for 20 h. After extensive washes, bound GR was eluted with 2× sample buffer, analyzed on 10% SDS−PAGE gels, and detected by autoradiography as described in the Materials and Methods. Similar results were obtained for each GMEB-2 chimera in two to six additional experiments. (C) The amount of each GST/GMEB-2 chimera that was immobilized on the anti-GST matrix was directly determined by Western blotting with anti-GST antibody of the material eluted from the matrix with 2× sample buffer following separation on 10% SDS−PAGE gels (positions of molecular weight markers [kDa] are indicated on the left). For each GST/GMEB2 construct, the desired full-length chimera is the largest and most abundant protein, with the smaller species representing C-terminally truncated fragments that still retain the epitope for the anti-GST antibody.

*GMEB-2 Domain for Interaction with CBP*. The precise mechanism by which the GMEBs affect the position of the dose−response curve of GR−agonists, and the partial agonist activity of GR−antagonists, is still unclear (*20*, *24*, *37*). However, one transcriptional cofactor that is known to associate with the GMEBs is CBP. In fact, the interactions of the C-terminus of CBP (amino acids 1678−2441) appear to be stronger for GMEB-2 than for GMEB-1 (*20*). These interactions were examined in greater detail using the same mammalian two-hybrid assay, this time with GAL-DBD/CBP and the VP16/GMEB-2 mutants of Figure 7A. Again, a large segment of GMEB-2 is essential for strong interactions with CBP (Figure 7B). Attempts to reduce the amount of GMEB-2 to less than amino acids 47−309 consistently caused diminished responses even though all proteins were expressed at about the same level (Figure 7C).

*Interaction Domains of GMEB-1 and -2 with Ubc9*. A recently identified component of the modulatory activity of the GMEBs is Ubc9, which is a human homologue of the E2 ubiquitin-conjugating enzymes of yeast. Ubc9 was identified by its ability to bind to GMEB-1 in a yeast two-hybrid screen. Ubc9 was found to bind GMEB-2 less avidly and to also bind to GR (*25*). Our earlier study on the activity domains of GMEB-1 (*24*) did not examine the region(s) required for binding to Ubc9. Therefore, we analyzed here the sequences of both GMEB-1 and GMEB-2 that are needed for binding to Ubc9, as determined by pulldown assays with a GST/Ubc9 chimera and [$^{35}$S]methionine-labeled, in vitro translated GMEBs. For GMEB-1, the major binding domain is defined by amino acids 1−306. This domain appears to be composed of two sites (1−171 and 177−306), of which the former is the strongest (Figure 8). However, no consistent pattern is observed for the binding of the GMEB-2 fragments to Ubc9 (data not shown). Presumably, this is due, in large part, to the weaker binding of Ubc9 to GMEB-2 than to GMEB-1 that was initially reported (*25*).

*Domains Responsible for Apparent Increased Molecular Mass of GMEB-2*. A common feature of both GMEB-1 and GMEB-2 is that the calculated molecular mass of the cloned proteins was much smaller than the apparent mass of the protein on SDS gels (*19*, *20*). For GMEB-2, the observed and calculated masses are 67 versus 56.5 kDa, respectively. Three regions of GMEB-2 appear to contribute to this apparent increased mass: 1−46, 185−309, and 368−423 (data not shown). The fact that the total increased apparent mass is less than that of the individual components suggests that the contributions of each segment are not additive and are somehow attenuated in the full-length protein.

## DISCUSSION

GMEB-1 and -2 exist as a heterooligomer both in solution and upon binding to the GME of the rat tyrosine aminotransferase gene (*18*, *20*). We previously reported on the structure/activity relationships for GMEB-1 (*24*). Here, we determine the regions of GMEB-2 that are required for the various activities of GMEB-2 (Figure 9). Many of the activities require extensive stretches of the GMEB-2 protein. This is not due to a lack of appropriate constructs to more precisely define the boundaries, although additional constructs should be able to slightly restrict the domains. Instead, the requirement for large regions of GMEB-2 often reflects the composite nature of the particular property. For example, a relatively small sequence of 58 amino acids is sufficient to encode most of the intrinsic transactivation activity of GMEB-2. However, more complicated processes, such as the DNA binding of a homooligomer, minimally requires both the homooligomerization domain of amino acids 185−309 and the KDWK motif at position 140 (Figure 4). The need of residues more C-terminal of the homooligomerization domain and more N-terminal of the KDWK motif undoubtedly reflect the additional elements needed to maintain a unique surface of the protein as opposed to a contiguous sequence of amino acids. Other features are less obvious and will require further investigations. For example, it is not clear why such a large surface is necessary for the binding of CBP (Figure 7) when other proteins, such as the coactivator TIF2, employ only about 60 amino acids (*38*, *39*). We note that GMEB-2/N309 contains the KDWK sequence that is essential for DNA binding and the regions necessary for oligomerizing and for interacting with GR and CBP. However, species not extending to position 369 are inactive both for DNA binding and for modulating the $EC_{50}$ of GR−agonist complexes, and the partial agonist activity of GR−antagonist complexes. Thus, residues between 310 and 369 are critical for several unidentified functions that are required for the modulation of GR transcriptional properties. It is also interesting that homooligomerization involves residues in addition to those of amino acids 214−309 that are sufficient for heterooligomerization (Figures 2 and 3). An X-ray structure of this complex would be very helpful in answering

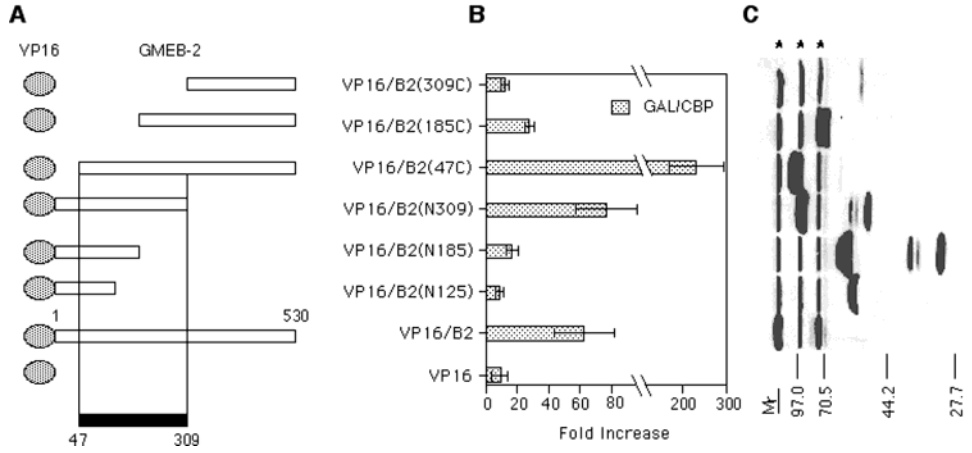


FIGURE 7: CBP interaction domain of GMEB-2. (A) Structure of the VP16/GMEB-2 chimeras used in the two-hybrid assay. (B) Biological activities of VP16/GMEB-2 chimeras in two-hybrid assay with GAL/CBP. Duplicate samples of COS-7 cells were transiently transfected as described in the Materials and Methods with 1 $\mu$g each of the indicated VP16/GMEB-2 chimeras and reporter (GAL4-E1B-LUC), 1 $\mu$g of either GAL or GAL/CBP chimera plasmid, and 10 ng of Renilla plasmid as an internal control. The relative Luciferase activities were normalized for Renilla activity. The fold increase in Luciferase activity caused by the presence of CBP was then determined by dividing the normalized activity of each VP16/GMEB-2 construct with GAL/CBP by the normalized activity of the same VP16/GMEB-2 construct with GAL. The average values from two to four experiments (±SEM) are plotted. (C) The expression levels of the VP16/GMEB-2 chimeras after transient transfection in COS-7 cells was determined by Western blotting with anti-VP16 antibody (positions of molecular weight markers [kDa] are indicated on the left). Asterisks (*) indicate nonspecifically detected bands.

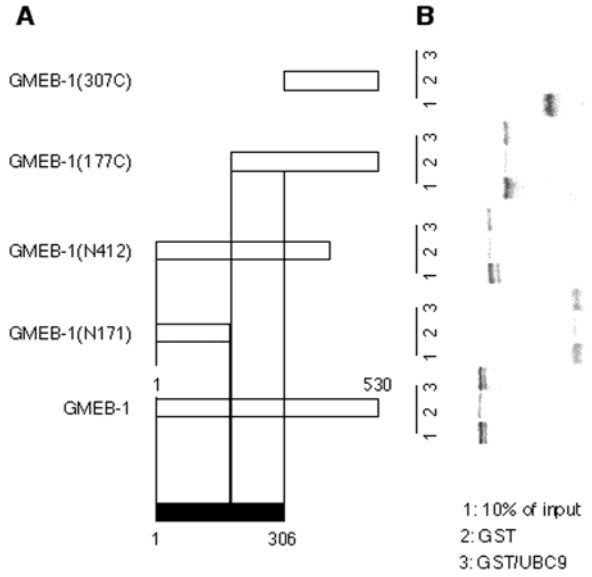


FIGURE 8: Ubc9 interaction domain of GMEB-1. (A) Structure of the in vitro translated forms of GMEB-1 that were used in the pulldown assay. (B) Immobilization of GMEB-1 fragments by Ubc9 in a pulldown assay. Bacterially expressed GST or GST/Ubc9 chimera, which had been immobilized on glutathione-Sepharose beads, were incubated with in vitro translated, [$^{35}$S]methionine-labeled forms of GMEB-1 for 20 h. After extensive washes, the bound GMEB-1s were eluted with 2× sample buffer, analyzed on 10% SDS−PAGE gels, and detected by autoradiography (lanes labeled 3) as described in the Materials and Methods. Nonspecific binding to GST corresponds to lanes labeled 2. Lanes labeled 1 contain 10% of the [$^{35}$S]methionine-labeled GMEB-1s that was incubated with the beads containing GST or GST/Ubc9. Similar results were obtained in two additional experiments.

these questions. No property investigated requires the C-terminal 100−150 amino acids of GMEB-2. It remains to be seen whether this region is involved in other yet unidentified activities related to GR transactivation or apparently unrelated properties, such as parvovirus replication (*33*, *34*) and the activation the viral nickase NS1 that is a property of the GMEB-2 homooligomer (*35*).

The GMEB-2 domains encoding each of the activities examined in this study have approximately the same location in the primary protein sequence as in GMEB-1. The main exception is the intrinsic transactivation domains of GMEB-1 and -2, which are at opposite ends of each protein (Figure 9, bottom) (*24*). This degree of conserved functional organization might not have been expected because the two proteins are only 39% homologous and are encoded by genes on two different chromosomes (*21*). However, the previously noted higher level (55%) of cDNA homology (*20*) and extensive conservation of genomic organization, which even extends to portions of some introns, had suggested that both GMEBs evolved from a single parent gene (*21*). The very similar functional organization observed here further supports this hypothesis.

The physical separation of the intrinsic transactivation activity from all other activities in both GMEB-2 (Figure 9) and GMEB-1 (*24*) is of mechanistic significance. The inclusion of those domains for simple processes in the larger domains for more complex phenomena is consistent with the interpretation that the simple processes are required for the more complex processes, such as GMEB oligomerization being required for the DNA binding of GMEBs. This inclusion of oligomerization domains has been seen for several other DNA-binding proteins, such as the d-box in second zinc finger of the DBD of steroid receptors (*40*) and the 300 bp Rel homology domain that dictates the DNA binding and homo- and heterodimerization of Rel/NF$\kappa$B family members (*41*). Conversely, the separation of the GMEB-2 domains that modulate the $EC_{50}$ of GR−agonist complexes, and the partial agonist activity of GR−antagonist complexes, from those required for the intrinsic transactivation activity of GMEB-2 suggests that the two activities involve unrelated processes. This same deduction was made for GMEB-1 (*24*), which has significantly less modulatory activity (*20*). The fact that both proteins of the naturally occurring heterooligomer (*18*) display a separation of modulatory versus transcriptional activities is consistent with

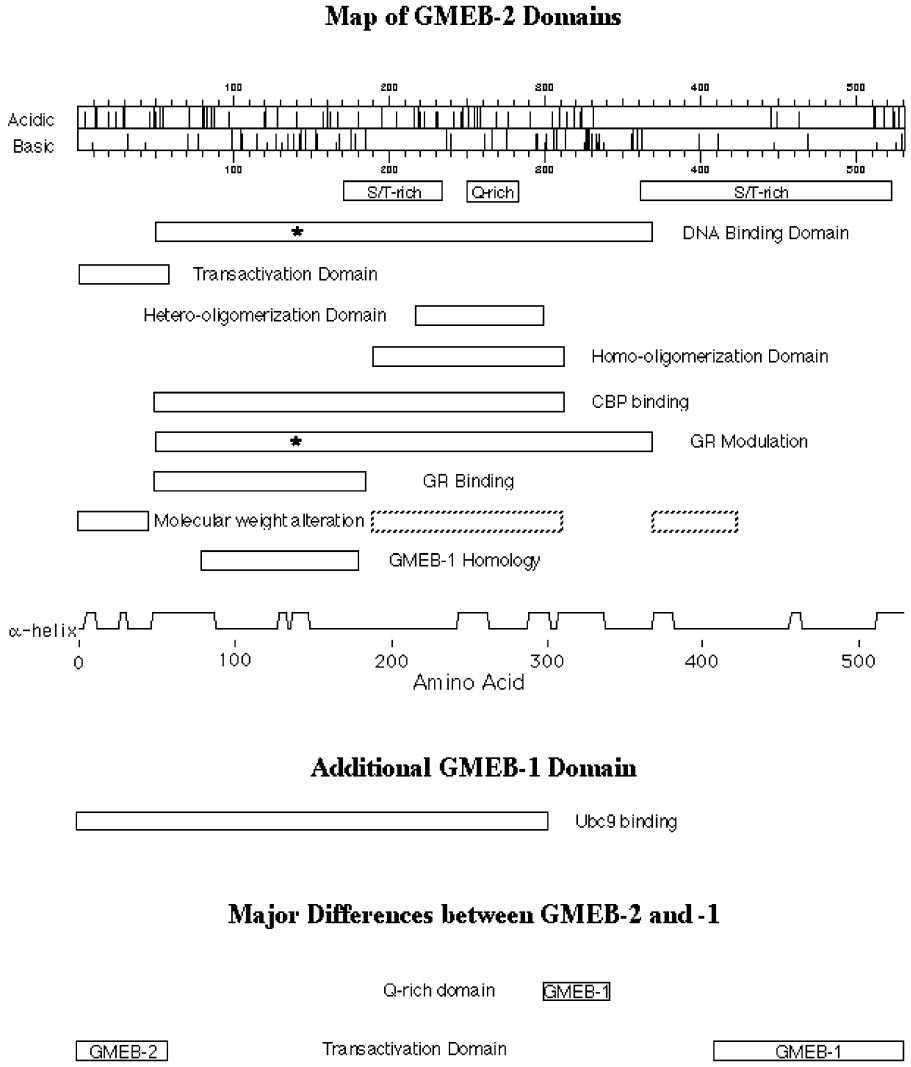


FIGURE 9: Comparison of domains of GMEB-2 and -1. (Top) summary of GMEB-2 domains. The acidic and basic regions of GMEB-2 were identified by DNA Strider. Q and S/T rich boxes are defined as sequences of 10 amino acids that are ≥30% glutamine or serine/threonine, respectively. The boundaries for the domains (depicted by rectangles) of the various indicated activities are those that have been determined by the above experiments. The asterisk indicates the position of the KDWK motif. Two of the domains for molecular weight have dashed borders to indicate that their contributions are less concentrated than that for the amino terminal sequence of amino acids 1−46. The domain for homology with GMEB-1 is taken from Kaul et al. (*20*). Potential α-helical secondary structures of GMEB-1 sequences were calculated using three programs (GCG, PHD <http://www.embl-heidelberg.de/predictprotein/predictprotein.html>, and NNPREDICT <http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html>). The displayed result was predicted by at least two of the three programs. (Middle) Additional domain of GMEB-1 that binds Ubc9. (Bottom) Major differences in positioning of domains of GMEB-2 and -1.

our earlier conclusions that the processes governing the total level of transactivation and the modulation of $EC_{50}$ and partial agonist activity are controlled by different mechanisms (*5*, *7*, *8*, *37*, *42*−*45*).

Many of the biological and biochemical activities of GMEB-2 are greater than those of GMEB-1. The DNA binding (*18*, *20*), intrinsic transactivation (*20*), ability to modulate GR transcriptional properties (*20*), and the interactions with CBP (*20*) are all more robust with GMEB-2 than with GMEB-1. The similar organization of all activity domains, except for intrinsic transactivation, in both proteins suggests that amino acid sequences, rather than the spatial organization of interacting domains, is responsible for the observed differences.

SAND domain proteins, like the GMEBs, contain a novel α/β-fold and a KDWK motif that is involved in DNA binding. These conclusions derived from an elegant NMR study of a fragment of NUDR (nuclear DEAF-1 related) (*23*), which is a mammalian transcriptional regulator. NUDR binds to DNA as a dimer to a tandem array of TTGC sites. However, the transcriptional activity of NUDR was suggested to occur independently of its DNA binding, perhaps via protein−protein interactions (*46*). This, plus the observation that a CGTC site is required for the DNA binding and gene activation by GMEB homo- and heterooligomers (*17*, *18*, *20*), raised the question as to whether the KDWK sequence of the GMEBs is actually required for DNA binding. The ability of the K140I,D141S mutation of this KDWK motif to eliminate the DNA binding of GMEB-2 (Figure 4) establishes the critical role of this sequence in the DNA binding of GMEB-2 and supports the earlier conclusion that this motif is important for the DNA binding of SAND domain proteins in general (*19*, *20*, *23*), even if the DNA sequence to which they bind is slightly different.

In summary, many of the domains encoding the ability of GMEB-2 to modulate GR transactivation properties have

now been defined. When combined with the data for GMEB-1 (*24*), the importance of different domains in the functioning of the naturally occurring heterooligomer can be assessed. We are now in a position to determine the interacting proteins that participate in the expression of several biological responses, such as the intrinsic transactivation activity and the GR modulatory activity. When similar identifications are available concerning the activities of the GMEBs in parvovirus replication, comparisons of the role of any domains with activities in both systems should provide additional information concerning the detailed mechanisms of action of these two proteins.

## ACKNOWLEDGMENT

We thank Otto Fröhlich, Nasreldin M. Ibrahim, and S. Russ Price for the generous donation of plasmids and Roland Owens (NIDDK, NIH) for critical review of this paper.

## REFERENCES

1. McKenna, N. J., Nawaz, Z., Tsai, S. Y., Tsai, M.-J. and O'Malley, B. W. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 11697−11702.
2. Underhill, C., Qutob, M. S., Yee, S. P. and Torchia, J. (2000) *J. Biol. Chem. 275*, 40463−40470.
3. Huang, E. Y., Zhang, J., Miska, E. A., Guenther, M. G., Kouzarides, T. and Lazar, M. A. (2000) *Genes Dev. 14*, 45−54.
4. Li, J., Wang, J., Nawaz, Z., Liu, J. M., Qin, J. and Wong, J. (2000) *EMBO J. 19*, 4342−4350.
5. Szapary, D., Huang, Y., and Simons, S. S., Jr. (1999) *Mol. Endocrinol. 13*, 2108−2121.
6. Giannoukos, G., Szapary, D., Smith, C. L., Meeker, J. E. W., and Simons, S. S., Jr. (2001) *Mol. Endocrinol. 15*, 255−270.
7. Song, L.-N., Huse, B., Rusconi, S., and Simons, S. S., Jr. (2001) *J. Biol. Chem. 276*, 24806−24816.
8. He, Y., Szapary, D., and Simons, S. S., Jr. (2002) *J. Biol. Chem. 277*, 49256−49266.
9. Simons, S. S., Jr. (2003) *TIPS 24*, 253−259.
10. Wang, Q., Blackford, J. A., Jr., Song, L.-N., Huang, Y., and Simons, S. S., Jr., submitted.
11. Wang, Q., Richter, W. F., Anzick, S. L., Meltzer, P. S., and Simons, S. S., Jr., under review.
12. Anzick, S. L., Azorsa, D. O., Simons, S. S., Jr., and Meltzer, P. S. (2003) *BMC Cancer 3*, 22.
13. Baulieu, E. E. (1989) *Science 245*, 1351−1357.
14. Vayssiere, B. M., Dupont, S., Choquart, A., Petit, F., Garcia, T., Marchandeau, C., Gronemeyer, H., and Resche-Rigon, M. (1997) *Mol. Endocrinol. 11*, 1245−1255.
15. Crawford, E. D., Eisenberger, M. A., McLeod, D. G., Spaulding, J. T., Benson, R., Dorr, F. A., Blumenstein, B. A., Davis, M. A., and Goodman, P. J. (1989) *N. Engl. J. Med. 321*, 419−424.
16. Jordan, V. C., and Murphy, C. S. (1990) *Endocr. Rev. 11*, 578−610.
17. Oshima, H., and Simons, S. S., Jr. (1992) *Mol. Endocrinol. 6*, 416−428.
18. Oshima, H., Szapary, D., and Simons, S. S., Jr. (1995) *J. Biol. Chem. 270*, 21893−21910.
19. Zeng, H., Jackson, D. A., Oshima, H., and Simons, S. S., Jr. (1998) *J. Biol. Chem. 273*, 17756−17762.
20. Kaul, S., Blackford, J. A., Jr., Chen, J., Ogryzko, V. V., and Simons, S. S., Jr. (2000) *Mol. Endocrinol. 14*, 1010−1027.
21. Zeng, H., Kaul, S., and Simons, S. S., Jr. (2000) *Nucleic Acids Res. 28*, 1819−1829.
22. Gross, C. T., and McGinnis, W. (1996) *EMBO J. 15*, 1961−1970.
23. Bottomley, M. J., Collard, M. W., Huggenvik, J. I., Liu, Z., Gibson, T. J., and Sattler, M. (2001) *Nat. Struct. Biol. 8*, 626−633.
24. Chen, J., Kaul, S., and Simons, S. S., Jr. (2002) *J. Biol. Chem. 277*, 22053−22062.
25. Kaul, S., Blackford, J. A., Jr., Cho, S., and Simons, S. S., Jr. (2002) *J. Biol. Chem. 277*, 12541−12549.
26. Theriault, J. R., Charette, S. J., Lambert, H., and Landry, J. (1999) *FEBS Lett. 452*, 170−176.
27. Paul, C., Manero, F., Gonin, S., Kretz-Remy, C., Virot, S., and Arrigo, A. P. (2002) *Mol. Cell. Biol. 22*, 816−834.
28. Garrido, C., Fromentin, A., Bonnotte, B., Favre, N., Moutet, M., Arrigo, A. P., Mehlen, P., and Solary, E. (1998) *Cancer Res. 58*, 5495−5499.
29. Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P., and Gronemeyer, H. (1996) *EMBO J. 15*, 3667−3675.
30. Jimenez-Lara, A. M., Heine, M. J. S., and Gronemeyer, H. (2000) *FEBS Lett. 468*, 203−210.
31. McElhinny, A. S., Kakinuma, K., Sorimachi, H., Labeit, S., and Gregorio, C. C. (2002) *J. Cell Biol. 157*, 125−136.
32. GenBank Accession No. BD186249.
33. Christensen, J., Cotmore, S. F., and Tattersall, P. (1997) *J. Virol. 71*, 5733−5741.
34. Christensen, J., Cotmore, S. F., and Tattersall, P. (1999) *Mol. Cell. Biol. 19*, 7741−7750.
35. Christensen, J., Cotmore, S. F., and Tattersall, P. (2001) *J. Virol. 75*, 7009−7017.
36. Simons, S. S., Jr., and Thompson, E. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3541−3545.
37. Zeng, H., Plisov, S. Y., and Simons, S. S., Jr. (2000) *Mol. Cell. Endocrinol. 162*, 221−234.
38. Voegel, J. J., Heine, M. J. S., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998) *EMBO J. 17*, 507−519.
39. Ma, H., Hong, H., Huang, S.-M., Irvine, R. A., Webb, P., Kushner, P. J., Coetzee, G. A., and Stallcup, M. R. (1999) *Mol. Cell. Biol. 19*, 6164−6173.
40. Freedman, L. P. (1992) *Endocr. Rev. 13*, 129−145.
41. Gilmore, T. D., and Morin, P. J. (1993) *Trends Genet. 12*, 427−433.
42. Oshima, H., and Simons, S. S., Jr. (1993) *J. Biol. Chem. 268*, 26858−26865.
43. Szapary, D., Xu, M., and Simons, S. S., Jr. (1996) *J. Biol. Chem. 271*, 30576−30582.
44. Chen, S., Sarlis, N. J., and Simons, S. S., Jr. (2000) *J. Biol. Chem.* 275, 30106−30117.
45. Kaul, S., Murphy, P. J. M., Chen, J., Brown, L., Pratt, W. B., and Simons, S. S., Jr. (2002) *J. Biol. Chem. 277*, 36223−36232.
46. Huggenvik, J. I., Michelson, R. J., Collard, M. W., Ziemba, A. J., Gurley, P., and Mowen, K. A. (1998) *Mol. Endocrinol. 12*, 1619−1639.

BI035311B