

Genomic organization of human GMEB-1 and rat GMEB-2: structural conservation of two multifunctional proteins

Huawei Zeng, Sunil Kaul and S. Stoney Simons Jr*

Steroid Hormones Section, Building 8, Room B2A-07, NIDDK/LMCB, National Institutes of Health, Bethesda, MD 20892, USA

Received November 23, 1999; Accepted February 13, 2000

DDBJ/EMBL/GenBank accession nos[†]

ABSTRACT

The glucocorticoid modulatory element binding proteins 1 and 2 (GMEB-1 and GMEB-2) are of interest both for their multiple activities (e.g. modulation of transactivation by the glucocorticoid receptor and initiation of parvovirus replication) and their membership in the emerging family of KDWK proteins. The genomic sequence of these proteins was desired in order to begin studies on the control of GMEB expression and to pursue previous evidence for significant homologies between the GMEBs. We now report the genomic sequence of human GMEB-1 and rat GMEB-2. The structure of both genes, including portions of the introns, is highly conserved. However, GMEB-1 and GMEB-2 were found to reside on chromosomes 1 and 20, respectively, demonstrating that they are encoded by distinctly different genes. Several isoforms of the GMEBs have been reported or detected in this study, and the splicing patterns were determined. The tissue distribution of each GMEB is not the same and is highest in fetal and developing tissues, consistent with previous suggestions that both homo- and hetero-oligomers may possess biological activity. The promoter region of both genes has been identified and both display high levels of transcription activity in transiently transfected cells when fused upstream of a promoterless reporter. These results indicate that the GMEBs are proteins that evolved from a single parent gene, have been highly conserved since the divergence of rats and humans and probably play important roles in development and differentiation.

INTRODUCTION

A new family of nuclear proteins sharing a KDWD domain (1) is currently emerging (2–8; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication).

Perhaps the most extensively studied members of this family are two proteins called glucocorticoid modulatory element binding proteins 1 and 2 (GMEB-1 and GMEB-2) (2), or p96 and p79 (7). The GMEBs were originally examined for their role in modulating the properties of glucocorticoid receptor-mediated transactivation (9–11). More recently, it was found that GMEB-1 and GMEB-2 are almost certainly the same as p96 and p79 (2,3,7), which are known to be essential auxiliary factors for the replication of parvoviruses (12). Another recent study reported that GMEB-1 associates with heat shock protein 27 (hsp27) (8), thereby implicating the GMEBs in yet other cellular functions. The fact that the GMEBs possess multiple activities suggests that they are highly conserved proteins with major roles in development and differentiation.

Our focus for several years has been on the GMEBs, which bind to a DNA transcriptional element called a glucocorticoid modulatory element (GME). This element has the novel properties of affecting two key parameters in steroid hormone action: the position of the dose–response curve of agonists and the magnitude of the partial agonist activity of antagonists (2,3,6,11). The dose–response curve defines the ligand concentrations at which the transcriptional activity of a given receptor is most sensitive to hormone. The amount of partial agonist activity of a given antisteroid is of major importance in the endocrine treatment of various disease states.

The GME was identified as a 21 bp element of the rat tyrosine aminotransferase (TAT) gene that is responsible for the different induction properties of this gene versus other glucocorticoid-inducible genes in the same cell (9,10,13,14). The GME is active in stably (15) and transiently transfected cells (11) with homologous and heterologous enhancers, promoters and genes (11,16). In the context of the TAT gene, the GME acts in conjunction with both a negative (17) and a neutralizing (16) element. However, when placed within 250 bp upstream of a tandem repeat of GREs, the 21 bp GME element is able to reproduce all of the effects of the intact TAT gene sequences (11,16,18).

The GME is especially active at physiological concentrations of agonist steroids (19). This means that the presence of a GME element *in cis* can significantly increase the cellular response of a given gene to glucocorticoid hormones relative to a gene

*To whom correspondence should be addressed. Tel: +1 301 496 6796; Fax: +1 301 402 3572; Email: steroids@helix.nih.gov

[†]AF203692, AF203693, AF205778–AF205780

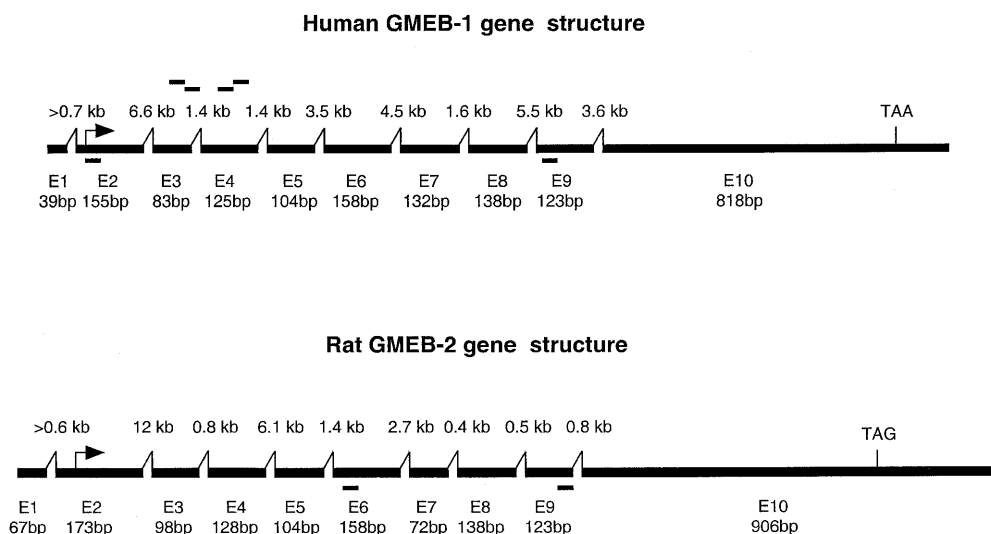


Figure 1. Genomic structure of the hGMEB-1 and rGMEB-2 genes. The 10 exons (E1, E2, etc.) comprising the cDNA for hGMEB-1 and rGMEB-2 are depicted by the thick solid line. The presence of an intron is designated by the thin sawtooth line. The length of each exon (intron) is given below (above) the structure. The filled bars above each sequence indicate the primers used to locate the chromosome containing each gene; the bars below the sequence represent the primers used to determine the tissue distribution of GMEB mRNAs (see also Fig. 3 for primers for tissue location of hGMEB-2). The bent arrow indicates the start of translation. The locations of the stop codons (TAA and TAG) are indicated.

lacking the GME (9,10,13,14). Thus, the existence of the GME offers a mechanism by which differential control of gene expression can be achieved with the same steroid-receptor complex. In addition, the activity of a GME-containing gene is not static but can be further modified by cell growth conditions (11,20). This suggested that the abundance and/or activity of a *trans*-acting factor might be modified to enable further control of the properties of glucocorticoid receptor (GR)-inducible genes.

Direct evidence for the involvement of a *trans*-acting factor in GME action was obtained from the correlation between biological activity of wild-type and mutant GME oligonucleotides and the ability of nuclear extracts from a variety of cells to bind to the same oligonucleotides in a gel shift assay (11). Conventional oligo-affinity chromatography was used to isolate two proteins of apparent molecular weights 88 (GMEB-1) and 67 (GMEB-2) kDa that existed in cells as a stable heteromeric complex of ~600 kDa (2). Both proteins have been cloned, although from different species. The rGMEB-2 clone was obtained by PCR of rat mRNA using degenerate oligonucleotides (3) derived from sequenced rGMEB-2 peptides (2). This same approach did not succeed with rGMEB-1, but careful examination of existing ESTs for matches with six rGMEB-1 peptides did reveal one human candidate that led to the eventual cDNA clone (2,6). The cloned proteins could, like the purified GMEBs, be recombined to afford the native DNA-binding complex (3,6). GMEB-1 and GMEB-2 were each found to be novel proteins that possessed intrinsic transactivation activity and may be members of a new family of proteins (3; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication).

Given the complexity of GME action and the roles of the GMEBs in other cellular functions (7,8,12), we were interested

in determining the genomic sequences of these proteins. Furthermore, similarities between GMEB-1 and GMEB-2 suggested that the genomic sequences might be highly homologous. Despite species differences between the human GMEB-1 clone (6) and the rat GMEB-2 clone (3), the two proteins are 39% identical at the amino acid level, with one region of 93 amino acids being 80% conserved (S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr submitted for publication). Each protein binds to itself and the other protein to form homo- and hetero-oligomers. They all display the same sequence specificity for DNA binding. They both have intrinsic transactivation activity. Finally, the GR binds to each of the GMEBs, using immobilized FLAG-tagged GMEBs in a standard pull-down assay, and with the C-terminal half of CBP in a mammalian two-hybrid assay (S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication). These results suggested that the GMEBs are highly conserved, perhaps due to some essential role during development.

The purpose of this study was to address a number of questions raised by the above observations. Are the GMEBs products of the same or different genes? How do the GMEBs compare at the genomic level with regard to homology, organization and chromosomal location? What is the tissue distribution of the GMEBs? Is the ratio GMEB-1:GMEB-2 the same in each tissue? Answers to this question could shed light on whether the activity of GMEBs in cells might be achieved with homo-oligomers or requires hetero-oligomers. Finally, are the GMEB promoters responsive to changes in cell culture or glucocorticoid steroids, thereby altering the levels of GMEB transcription? We now describe the genomic sequence of each protein. This and other results allow us to address all of the above questions.

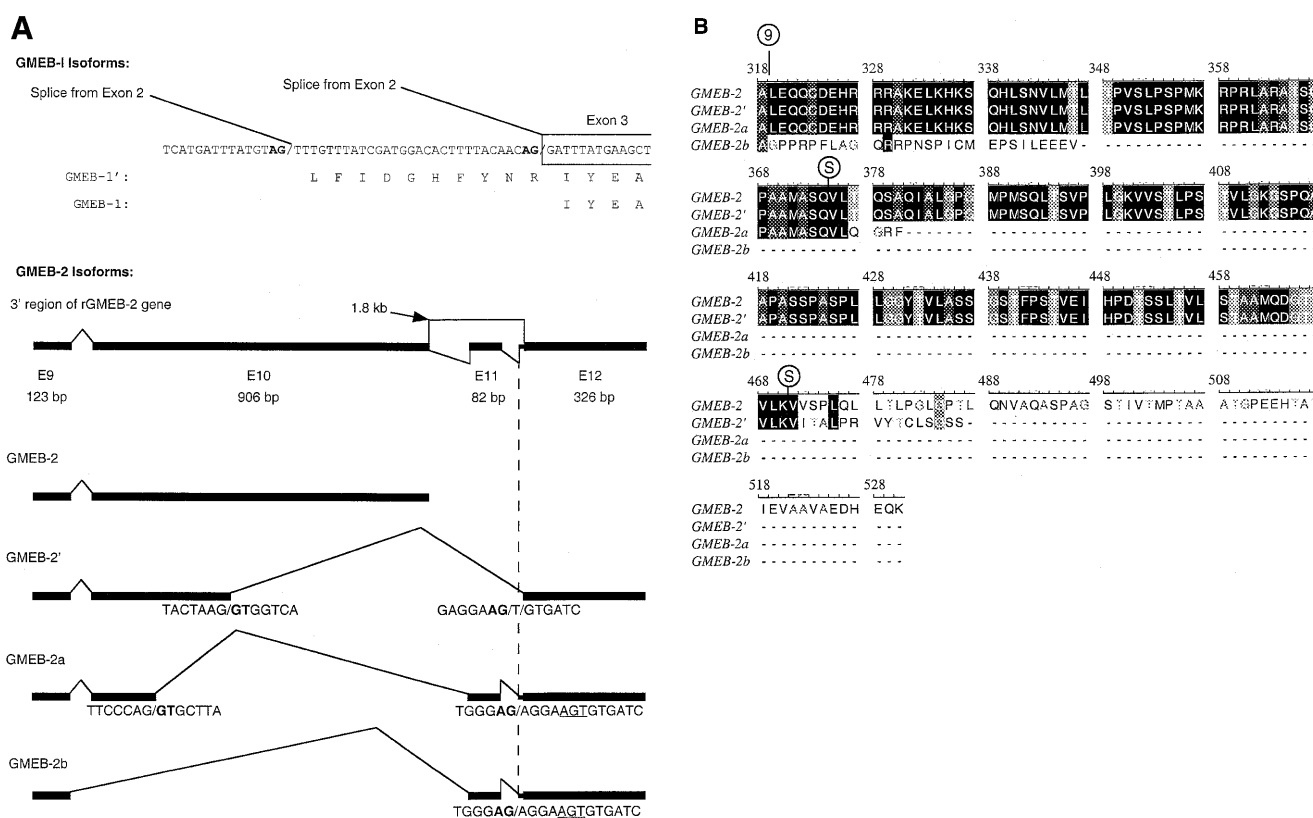


Figure 2. GMEB isoforms. (A) Structural differences between GMEB-1 and the longer GMEB-1' and between the four forms of GMEB-2. The indicated extra 10 amino acids of GMEB-1' (8) are shown to correspond to utilization of the 30 3' nucleotides of intron 2 that are recruited due to utilization of an alternative acceptor site (bold) in intron 2. With GMEB-2, the presence of two additional exons (E11 and E12, with lengths indicated below) were detected by PCR analysis of the genomic sequence. The precise location of E11 was not determined but is within the 1.8 kb sequence between E10 and E12. The different modes of GMEB-2 gene splicing that give rise to each isoform are indicated, with the exon/intron junctions inside exon 10 and at the edge of exon 12 given below the sequence. The junctions for splicing to and from exon 11 have not been determined. Note that the exon 12 acceptor splice site for GMEB-2a and GMEB-2b is 4 nt upstream of that used for GMEB-2' (underlined nucleotides). (B) C-terminal amino acid sequence of GMEB-2 isoforms. The amino acid sequence of each isoform is identical through Ala318 at the end of exon 9 (indicated by the circled 9), which is where alternative splicing affords GMEB-2b. The other isoforms result from splicing at sites indicated by the circled S to give the listed sequences.

MATERIALS AND METHODS

Cloning of hGMEB-1 and rGMEB-2 genes

The full-length hGMEB-1 cDNA (6) was used to obtain two hGMEB-1 genomic clones through the P1 human hybridization screening services of Genome Systems Inc. (St Louis, MO). The rGMEB-2 primers 5'-CCTCAAGCTGCCCCAGCCAGTT-3' (bp 1352-1373) and 5'-ACATTCTGCCCCCTTTTCTTCTCTT-3' (complementary to bp 1857-1881) were used to select two rGMEB-2 genomic clones through the P1 RAT PCR library screening services of Genome Systems Inc.

Tissue distribution

PCR amplification was performed on a Human Rapid-Scan, which contained first strand cDNA prepared from 24 human tissues (OriGene Technologies, Rockville, MD) with β -actin as an internal control. The PCR primers for hGMEB-1 were 5'-ATGGCTAATGCAGAAGTGAGTGT-3' and 5'-GTGTG-TGCTACATTGTTGAGAAC-3'. Initially, the rGMEB-2 primers (5'-GAGCTAGACTTCTACCAGCATGACAA-3' and 5'-TGCCAGGTCCCGTGCATACT-3') were used to amplify

a fragment from the above human tissue bank. The amplified band was then sequenced to ascertain its identity as human GMEB-2. Using this sequence, new PCR primers for hGMEB-2 (5'-GGTCTGCTCCAACACCTGCC-3' and 5'-TCCATCTG-GCACTTGTGGCT-3') were used to determine the presence of hGMEB-2 in different tissues.

Chromosomal localization

PCR amplification was performed on a panel of somatic cell hybrids that contained individual human chromosomes (Coriell Institute, Camden, NJ) using sets of nested exon primers constructed such that the amplified product crossed intron 3 and intron 8 for hGMEB-1 and hGMEB-2, respectively. The first and second set of nested PCR primers for hGMEB-1 were 5'-ACTCACACGATACACAAAATTGA-3'/5'-CATA-CAAATTCTTCCAGAGGAG-3' and 5'-GAAGAAGGGATT-GGTAAGGGTT-3'/5'-GATGGCTTTGCTCTCCCCACA-3'. The first and second set of primers for hGMEB-2 were 5'-GTCATCCAGGAGTTCCACCA-3'/5'-TCCATCTGGCA-CTTGTGGCT-3' and 5'-GTCATCCAGGAGTTCCACCA-3'/5'-GCCTGCACCTTCTTACCAG-3'. The resulting products

Table 1. Intron/exon junctions in GMEB-1 and GMEB-2 genomic sequences

GMEB-1 and GMEB-2 gene Structure				
Exon	Length	Donor site	Intron length	Acceptor site
<i>Human GMEB-1 gene</i>				
E1	39 bp	CCGCCGCCCGACGgAGACG/ GT GAGGAGGGG-->	0.7 kb --	TCCCAGCAG CAG /TCCCAGCTATCTGACTTCAT
E2	155 bp	TTACAGCCTGTGCAACAAGG/ GT AAGTGGCTAG--	6.6 kb --	CTTTTACAAC CAG /GATTTATGAAGCTGGGTCGG
E3	83 bp	CAAAATGAAGAAGGGATTG/ GT AAGGTTT--	1.4 kb --	TCTGTTT CAG /ATACAGGCATATAGAAGCA
E4	125 bp	TAAACGTGAAGTGTGTCAAG/ GT AATTGCTTT--	1.4 kb --	T TTTACTTTTAG /TTCAATGATCAGTTGATCAG
E5	104 bp	CTGGTGGGATCATGCTCAG/ GT AAGCTCTAAT--	3.5 kb --	CCCTTGCACAG /GAAAATGATGGACTCCGGAC
E6	158 bp	GACACCCACTTCGGCTGATG/ GT AGGGGTAGG--	4.5 kb --	GACTGTT TTTAG /GTAGCATCAGCAGATTGCC
E7	132 bp	AGACTCAGAGAAATTCAG/ GT ATTCTTCTAT--	1.6 kb --	TCTGTGTCC CAG /AGGACACTTTGATGTTCTGG
E8	138 bp	GGCTCCCTTCCAAGTCACAG/ GT AAGTGCCTA--	5.5 kb --	TGTTTTAAAT TAG /ATGCTGCTGTCTCAACAAT
E9	123 bp	GTCTCTATACTCTGCAG/ GT ATCTATAAGT--	3.6 kb --	T TTTTCCATAG /ACTTGGAAACGACAGTTGGAG
E10	818 bp	ACTTGGAAACGCCAGTTGGAG-----poly A		
<i>Rat GMEB-2 gene</i>				
E1	67 bp	GCAGGCCCGTGTGGGCCAG/ GT AAGCGGCATC-->	0.6 kb --	TGTTTACTCT TAG /GACTTCAGACTCTGGAGAAG
E2	173 bp	ACGAACCTAGCTCCTCATGG/ GT AAGAGTTAAG--	12 kb --	TCTGTCTACC CAG /GGGTGATTTGACAGAAGATA
E3	98 bp	GCTCAAGGAAGCCGTGTAG/ GT AGGAAACCT--	0.8 kb --	GTCTGTGAGA AG /TGAAGATGGCTGAAGAGGGG
E4	128 bp	TCAATGTGAAATGTGTTAG/ GT GAGGGTCAAG--	6.1 kb --	GT TTCTTTGTAG /TACGATGAACATGTGATCAG
E5	104 bp	ATGAACGGCATCATGCTCAG/ GT GGGCATCTGA--	1.4 kb --	GCCTCTTACAG /GAAGATCATGGACTCTGGGG
E6	158 bp	CACACCAGCTGCAGCTGATG/ GT AGGTGCAAGA--	2.7 kb --	CCTFACC CACAG /TGAATGGCTCACCTGCCACC
E7	72 bp	CTGGACCACAACCATTTGGAG/ GT ATAATCCCTC--	0.4 kb --	TCTCCATCAT AG /ACGACACATTTGCATTTCTGG
E8	138 bp	CCGCCCTTACAGCTCCGAG/ GT GAGAGGGCT--	0.5 kb --	TGTCCTTT CAG /ATGCTGTCTCTCTCAATAAC
E9	123 bp	GTATGCACGGACCTGGCAG/ GT ATGCCAGCAG--	0.8 kb --	TGTTTGGTGCAG /CCCTGGAAACGACAGTGTGAT
E10	906 bp	CCCTGGAAACGACAGTGTGAT-----poly A		

The intron/exon junctions (indicated by /) were determined by PCR of the genomic clones with the appropriate primers as described in Materials and Methods. The lengths of the exons and introns were directly determined and are listed. Only the size of the first intron for each gene could not be precisely determined due to the very high GC content. The consensus donor and acceptor sites for each splice are indicated by the bold lettering. Identical sequences in the most conserved introns of GMEB-1 and GMEB-2 are underlined.

were separated in a 1% agarose gel and subjected to DNA sequencing with a Radiolabeled Terminator Cycle Sequencing Kit for confirmation (US Biochemical).

Determination of intron-exon boundaries and intron sizes

Genomic clones were sequenced with a Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Cleveland, OH) using GMEB cDNA primers. Intron sizes were determined by PCR amplification using the genomic clones as templates, with a long-distance polymerase [eLONGase Amplification System (Gibco BRL, Gaithersburg, MD) or GeneAmp XL PCR Kit (Perkin Elmer, Norwalk, CT)]. The resulting products were separated in a 1% agarose gel and subjected to DNA sequencing with the Radiolabeled Terminator Cycle Sequencing Kit for confirmation.

Analysis of promoter activity, cell culture and transient transfection

Fragments of genomic DNA containing sequences upstream of the 5'-untranslated region of GMEB-1 and GMEB-2 were generated by primer walking with the Radiolabeled Terminator

Cycle Sequencing Kit followed by PCR with *Pfu* enzyme (Promega, Madison, WI). The PCR fragments were cloned into the polylinker (*Sma*I site) of the pGL3 reporter vector (Promega, Madison, WI) and the orientation was confirmed by obtaining the predicted fragment size after PCR with insert and vector primers. The largest inserts from each GMEB gene were re-sequenced to confirm the accuracy of this untranscribed region. Fu5-5 rat hepatoma clone 27 cells (13) were grown in Richter's improved minimum essential medium (IMEM) supplemented with 10% heat-inactivated fetal calf serum (Biofluids, Rockville, MD). COS-7 and CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 5 and 10% heat-inactivated fetal calf serum, respectively. Cells were transiently transfected with plasmid DNA, OPTI-MEM I and lipofectamine reagent for 4 h and then placed in normal medium for 16 h 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 Berthold luminometer (Microlumat LB 96 P).

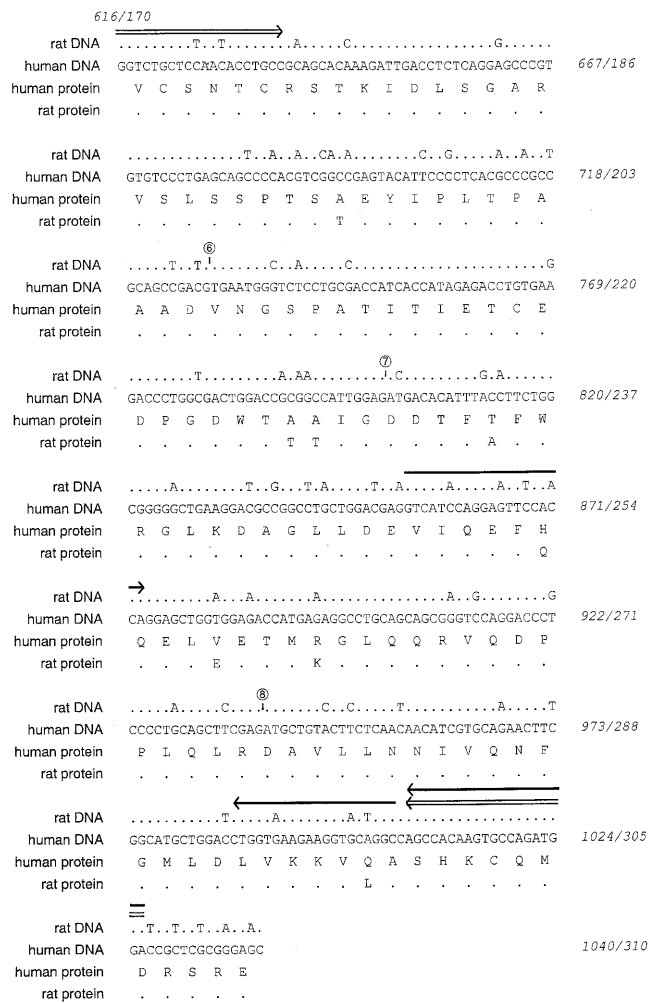


Figure 3. Comparison of the partial hGMEB-2 sequence with rGMEB-2. Differences in rGMEB-2 cDNA and predicted amino acid sequence (3) compared with those for the partial hGMEB-2 cDNA are listed above and below, respectively, the hGMEB-2 sequences (‘.’ indicates identity). Italicized numbers correspond to the nucleotide number of the rGMEB-2 cDNA clone followed by the amino acid number of the equivalent residue in full-length rGMEB-2 (3; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication). Circled numbers above the DNA sequence indicate the positions of introns in the genomic sequence of rGMEB-2 (Fig. 1). The primers used for tissue distribution and chromosomal location are marked by the double and thick single lines, respectively, above the DNA sequence.

RESULTS

Genomic structure of GMEB-1 and GMEB-2

hGMEB-1 genomic clones were obtained by hybridization to a human P1 library while rGMEB-2 genomic clones were selected by PCR screening of a rat P1 library (see Materials and Methods). In both cases, assorted primers from known cDNA clones (3,6) were used to obtain PCR products from the genomic clones. The PCR products were sequenced and the intron–exon junctions were determined. As shown in Figure 1, the genomic structure of the two genes is remarkably similar for different proteins from different species. Each gene has the identical number of introns and exons, with the first exon being untranslated. Furthermore, the exon lengths are not only very

Table 2. Comparison of tissue distributions of GMEB-1 and GMEB-2

		Expression	
Tissue		GMEB-1	GMEB-2
1	Brain	–	–
2	Heart	–	–
3	Kidney	–	–
4	Spleen	–	+
5	Liver	–	+
6	Colon	–	+
7	Lung	++	++
8	Small intestine	+	+
9	Muscle	+	++
10	Stomach	+++	+
11	Testis	+++	+++++
12	Placenta	++++	++++
13	Salivary gland	–	–
14	Thyroid gland	–	–
15	Adrenal gland	–	–
16	Pancreas	+	+
17	Ovary	–	+
18	Uterus	+	+
19	Prostate	+++	+
20	Skin	++	–
21	PBL	++	+++++
22	Bone marrow	++	+++
23	Fetal brain	+++++	–
24	Fetal liver	++++	+++

The relative intensity of the PCR-amplified band for the GMEB fragment in each tissue sample in Figure 2A and B was visually determined and then listed.

similar but also show the same variations in length as one proceeds down the gene. The intron length is, as expected, variable and does not display the coordinated changes in size as seen for the exons. Table 1 gives the precise sequence at all junctions and shows that each exon/intron junction contains consensus donor/splice junctions. The ends of several introns displayed ≥50% identity.

GMEB isoforms

One possible explanation for a high homology among the sequences of intron ends is that alternative splicing occurs. Thus, there would be selective pressure to maintain these sequences because they were occasionally being used as coding sequences. In fact, two alternative structures of both GMEB-1 (8) and GMEB-2 (3) have already been reported. As shown in Figure 2A, both of these species result from alternative splicing of the genomic sequences. A longer form of GMEB-1 (8), which we call GMEB-1’, appears to form by using an acceptor splice site that is 30 bases upstream of the start of exon 3. The formation of the shorter GMEB-2, initially called

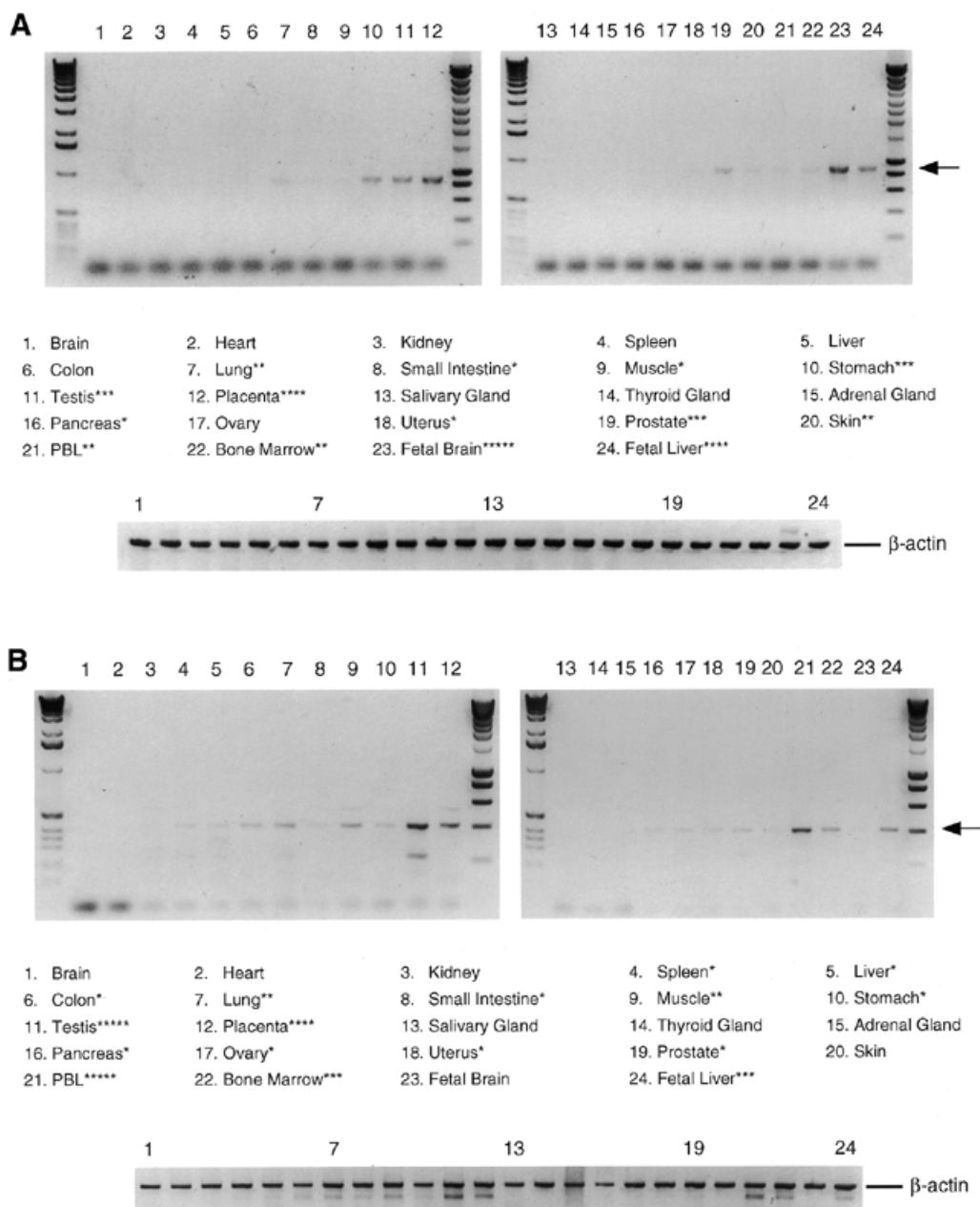


Figure 4. Distribution of GMEB-1 (A) and GMEB-2 (B) in human tissues. GMEB-1 and GMEB-2 specific fragments were amplified by PCR from pools of 24 different human tissue cDNAs as described in Materials and Methods. The arrow indicates the position of the common amplified fragment. The number of asterisks after each listed tissue represents the relative abundance of the particular GMEB being probed. Equal amplification of a β -actin fragment (shown below) confirms that the differences in amplified GMEB fragments were not due to unequal PCR amplification between different tissues.

GMEB-2' (3), is of interest in that it results from a cryptic splice site from within exon 10 of GMEB-2 to a new exon, exon 12, that is 1.8 kb downstream of exon 10. Consensus donor and splice sites are used (Fig. 2A). However, RNA editing (21) appears to have removed the first nucleotide of exon 12 from the message. Although exon 12 is 326 bp long, the resulting reading frame yields a premature termination to give a protein that is smaller than GMEB-2 (Fig. 2B). In addition to the GMEB-2' isoform, two other GMEB-2 variants (a and b)

have been detected by RT-PCR, each of which is also shorter than GMEB-2. Both of these isoforms contain an additional exon (exon 11) that is upstream of exon 12. Both of these isoforms also utilize an acceptor site which is 7 bases upstream of the start of exon 12 (Fig. 2A). GMEB-2a diverges from GMEB-2 at another cryptic splice site within exon 10, while GMEB-2b results from alternative splicing at the end of exon 9 (Fig. 2A). Both isoforms then splice to exon 11 and then the donor site upstream of exon 12. Even though both GMEB-2a

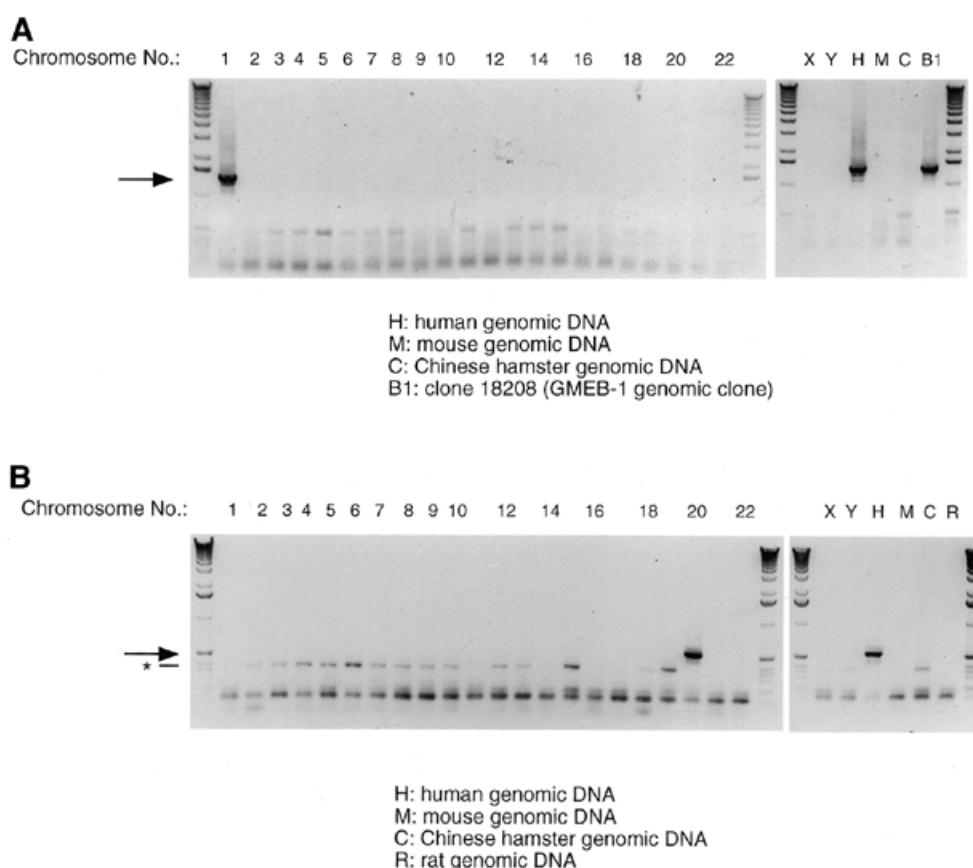


Figure 5. Chromosome location of GMEB-1 (A) and GMEB-2 (B). Correctly sized genomic fragments of hGMEB-1 or hGMEB-2 were amplified by two-cycle PCR as described in Materials and Methods. The arrow indicates the position of the correctly sized fragment, as confirmed by the appearance of the same species from the genomic clone [lane B1 in (A)] or from total human chromosomal DNA [lane H in (A) and (B)]. The * in (B) marks the position of a hamster GMEB-2 fragment (see lane C for control using total hamster chromosomal DNA) that was amplified from those cell samples that were of hamster origin.

and GMEB-2b share the same 3'-terminus (exon 11 and extended exon 12), the protein sequences are not the same due to different reading frames which give premature termination and shorter proteins than GMEB-2 (Fig. 2B).

Tissue distribution of GMEB-1 and GMEB-2

The detection of GMEB mRNA by northern blotting was difficult, perhaps due to very low concentrations of the messages. We therefore used PCR to determine the distribution of the GMEB transcripts in different human tissues. In each case, we used primers that spanned several introns (Fig. 1 and below), thereby eliminating problems emanating from contaminating genomic DNA sequences. The very high similarity in genomic organization of hGMEB-1 and rGMEB-2 (Fig. 1) plus the 92% identity between the amino acids of sequenced rGMEB-1 peptides (2) and the predicted amino acid sequence from the hGMEB-1 clone (6; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication) suggested that we could use primers from the rGMEB-2 exons to detect hGMEB-2 transcripts. In order to support this decision, the major band that was observed in RT-PCR screening of the human tissue bank with rGMEB-2 primers was amplified and sequenced to give a 425 bp oligonucleotide that encoded a

141 amino acid sequence (Fig. 3). This human sequence was highly homologous to our rGMEB-2 clone (85.2% identical at the DNA level and 94.3% identical at the amino acid level). We therefore conclude that this human sequence constitutes a partial sequence of hGMEB-2.

Primers from our human GMEB-1 (6; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication) and GMEB-2 (Fig. 3) cDNAs were then used to determine the presence of GMEB mRNAs in a variety of human tissues. In each case, β -actin was used as an internal control for PCR efficiency. As shown in Figure 4, the abundance of the GMEBs in numerous tissues is highly variable. Furthermore, there are many tissues in which the level of either GMEB was below detection. For both genes, there appears to be preferential expression in reproductive and/or developmentally important cells (e.g. testis, placenta, bone marrow and fetal tissues). In general, the relative expression levels of the two genes in each tissue are similar (Table 2), as might have been anticipated from the finding that GMEB-1 and GMEB-2 can exist as a heteromeric complex (2). However, each protein can form homo-oligomers (2; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication). The very high levels of GMEB-1 in fetal brain should be

Chromosomal location of GMEB-1 and GMEB-2

A commercially available bank of mouse and Chinese hamster cells, each containing one human chromosome, was used to determine the chromosomal location of the GMEB-1 and GMEB-2 genes. In each case, primers that spanned one intron were selected from our hGMEB-1 and partial hGMEB-2 clones to be used for PCR amplification of the genomic sequence (Figs 1 and 3). The authentic human gene and/or a complete human genomic DNA sample were used as positive controls. A single round of PCR afforded good positive control signals but was unable to yield detectable levels of an amplified species for either GMEB-1 or GMEB-2 from any of the cells. However, two rounds of PCR using nested primers gave a clean signal (Fig. 5). From this analysis, we conclude that GMEB-1 is on chromosome 1 while GMEB-2 resides on chromosome 20. This establishes that the GMEBs, although closely related, are different gene products.

No sequence was amplified by the GMEB-1 primers from either the mouse or hamster genomes (Fig. 5A). This is presumably due either to a lack of sufficient sequence homology between the human and mouse or hamster genes or to the presence of very large (or small) introns in the non-human species that are not correctly replicated (or are too small to be observed). It should be noted, however, that the GMEB-2 primers did amplify a smaller fragment from the hamster genome. This species (indicated by * in Fig. 5B) was observed only in samples that were, according to the supplier, derived from hamster cells.

Promoter region of the GMEB-1 and GMEB-2 genes

Previous results indicated an effect of cell density and culture conditions on the activity of the GME in Fu5-5 cells (11,20). These data suggested that cellular factors/processes, and perhaps added glucocorticoids, might act on the GMEB promoter sequences to regulate GMEB transcript levels. We therefore amplified the putative promoter region from each genomic clone (Fig. 6A) and determined the activity of different lengths when fused in front of a promoterless luciferase reporter (pGL3) in Fu5-5 cells (Fig. 6B). All promoter constructs displayed more activity than the SV40 promoter in Fu5-5 cells (Fig. 6C). This indicated that not only had we succeeded in isolating the promoter region of each GMEB gene but also that the promoter is very active in Fu5-5 cells.

An examination of the promoter sequences revealed one potential GRE half site in the GMEB-1 promoter and two possible half sites in the GMEB-2 promoter (Fig. 6A). The addition of the glucocorticoid dexamethasone (Dex) appeared to cause a weak increase in GMEB promoter activity. However, a similar weak effect was also seen with the SV40 promoter (Fig. 6C) and no increase in GMEB-2 protein was observed by western blotting in Fu5-5 cells exposed to 1 μ M Dex (data not shown).

In all cases, the activity of each GMEB reporter is eliminated when the promoter sequence was inserted in the inverse orientation. This suggests that some element(s) at the 3'-end of each sequence needs to be close to the start of transcription in order to convey good promoter activity. An attractive candidate for such a 3'-element is a GC-rich sequence that is immediately upstream of exon 1 in both the GMEB-1 and GMEB-2 genes.

When this 172 bp GC-rich sequence was deleted from two different GMEB-2 constructs, the resulting promoters were much less active (Fig. 6C). Thus, this 172 bp GC-rich sequence of the GMEB-2 gene, and probably of the GMEB-1 gene, is required for high activity of the promoter.

The importance of the 172 bp GC-rich sequence of the GMEB-2 promoter is retained in CV-1 and COS-7 cells (Fig. 6D). This lack of strict dependency on cell-specific factors for GMEB-2 promoter activity is consistent with expression of the GMEBs in multiple different tissues (Fig. 4). Interestingly, though, the strength of the GMEB promoters, relative to each other and to the SV40 promoter, does vary with cell type. The GMEB-1 promoter is usually more active than the GMEB-2 promoter in Fu5-5 and CV-1 cells while both are equally active in COS-7 cells. Compared to the SV40 promoter, both GMEB promoters are somewhat more active in Fu5-5 cells and less active in CV-1 cells. The much greater activity of the SV40 promoter in COS-7 cells is probably due to the presence of the SV40 T antigen in COS-7 cells, which will cause increased replication of the SV40-driven reporter plasmid.

The activity of the GME increases under conditions of low medium volume (11,20). In contrast, low volumes of medium decreased the activity of the GMEB-1 and GMEB-2 promoters (Fig. 6E), consistent with the observation that fresh medium caused higher levels of promoter activity than conditioned medium (Fig. 6C). However, similar effects were seen with the SV40 promoter (Fig. 6C and E). Therefore, we conclude that GMEB promoter activity is very high and can change in a cell-specific manner. However, the previously documented variations in GME activity under different growth conditions do not result from effects of glucocorticoid steroids or changes in the medium on GMEB promoter activity to modify GMEB protein levels.

DISCUSSION

GMEB-1 and GMEB-2 are two new proteins that are implicated in modulation of the dose-response curve for GR transactivation and of the partial agonist activity of anti-glucocorticoids (2,3,6,11,14-17; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication). The genomic organization and tissue distribution of GMEB-1 and GMEB-2 have now been determined. The tissue distribution of the GMEBs, determined by PCR of tissue-specific cDNA preparations, is highest in fetal and sex organs, suggesting an important role in development and differentiation. The mouse equivalent of GMEB-1 has been detected by western blotting in a wide variety of tissues, including brain, heart, kidney, spleen and liver (8). In contrast, none of these tissues from humans contained detectable amounts of GMEB-1 mRNA (Table 2). Whether this reflects species differences or a greater stability of the GMEB-1 protein compared to the mRNA remains to be investigated.

GMEB-1 and GMEB-2 were initially isolated as a stable heteromeric complex (2). However, they do homodimerize (2; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication; J.Chen, S.Kaul and S.S.Simons,Jr, in preparation), consistent with an independent action of each homo-oligomeric complex. The presence of GMEB-1, but not GMEB-2, in fetal human brain is consistent with such a hypothesis. Likewise, the apparently different

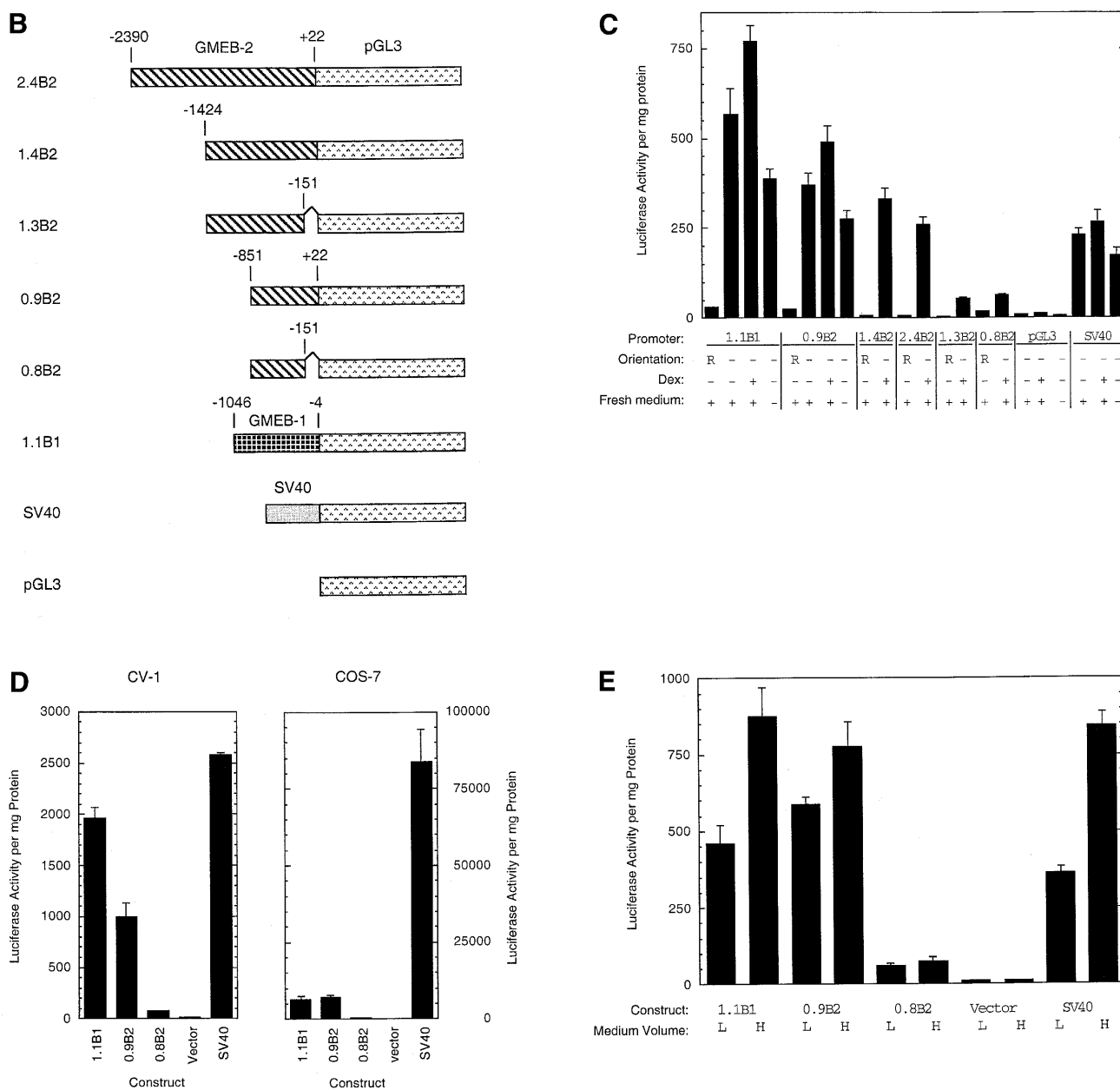


Figure 6. (Opposite and above) Transcriptional activity of GMEB-1 and GMEB-2 promoter sequences. (A) Promoter region sequences of the hGMEB-1 and rGMEB-2 genes. The start of transcription is indicated by the bent arrow. Possible GRE half-sites are boxed. The GC-rich region at the 3'-end of each sequence is underlined. (B) Diagrams depicting the different reporter constructs containing portions of the genomic sequences upstream of exon 1 for GMEB-2 (diagonal lines) or GMEB-1 (grid) fused to the promoterless reporter pGL3 (chevrons). As a control, a SV40 (gray shading) driven reporter was also used. Note that the sizes of the SV40 and pGL3 sequences are not to scale. (C) Activity of GMEB promoter sequences in Fu5-5 cells. The various constructs of (B), with the promoter sequence fused to the reporter plasmid in the correct or reverse (R) orientation, were transiently transfected into Fu5-5 cells in triplicate and incubated with and without added glucocorticoid (Dex) or fresh medium before determining the reporter activity as described in Materials and Methods. The averages \pm SD of a representative experiment were plotted. Similar results were obtained in two other experiments. (D) Activity of GMEB promoter sequences in CV-1 and COS-7 cells. Triplicate samples of the indicated plasmids were transiently transfected into CV-1 or COS-7 cells and incubated without added steroid but with fresh medium. The cells were harvested and the data plotted as for (B). Similar results were obtained in a second experiment. (E) Effect of tissue culture medium volume on GMEB promoter activity in Fu5-5 cells. Triplicate samples of the indicated plasmids were transiently transfected into Fu5-5 cells and incubated with 1.5 or 15 ml of medium. The cells were harvested and the data plotted as for (C). Similar results were obtained in a second experiment.

ratios of GMEB mRNA in several other tissues (Table 2) suggests unique activities of each GMEB. This speculation is further strengthened by the recent report that hGMEB-1 is co-immunoprecipitated with hsp27 (8).

The significant homology of both the DNA and amino acid sequences for GMEB-1 versus GMEB-2 suggests that these genes are closely related. This is now even more apparent when considering the genomic organization of the two genes.

Both the intron/exon structure and positioning within the gene are remarkably conserved (Fig. 1). Even more remarkable is that there appears to be significant conservation among some of the intron sequences, even though the two genes are from different species. The localization of GMEB-1 and GMEB-2 on human chromosomes 1 and 20, respectively, establishes that these proteins are encoded by different genes. However, the extensive sequence homologies, in addition to similar biological DNA binding properties (2,3; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication), oligomerization (2,3; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication) and transactivation and interactions with CBP and GR (S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication), strongly suggest that both proteins derived from a common ancestral gene. Interestingly, the homology between hGMEB-1 and rGMEB-2 is higher at the level of DNA sequence than protein sequence (55 versus 39%, respectively). The significance of this remains to be examined (22). Comparisons of the human and rat GMEB-2 proteins reveal the more common situation of a lower conservation of DNA than protein sequence (85 versus 95%). The maintenance of a high level of identity over the 80 million years since humans and rats diverged (22) is consistent with at least GMEB-2 being important in the development of both species.

As with many proteins, there are several isoforms of each GMEB (Fig. 2). A recently reported clone of hGMEB-1 contained a 10 amino acid insert (8) when compared to our hGMEB-1 (6; S.Kaul, J.A.Blackford,Jr, J.Chen, V.V.Ogryzko and S.S.Simons,Jr, submitted for publication). This insert, resulting from alternative splicing, was inside a sequenced tryptic peptide of rat GMEB-1 (2) and thus does not correspond to the major GMEB-1 isoform in rat. The three isoforms of GMEB-2 are each shorter than the full-length GMEB-2, with all of the changes arising from splicing variations after exon 9. Two of the isoforms (GMEB-2' and GMEB-2a) are formed by alternative splicing from within exon 10 (Fig. 2A). Splicing from within an exon is unusual but has been observed in other situations, such as formation of the human thyroid receptor $\alpha 1$ and $\alpha 2$ isoforms (23).

The GMEBs are not uniformly present in the tissues that were examined (Fig. 4). The high levels of mRNAs in fetal and reproductive tissues suggest key roles of these proteins in development and differentiation. Some tissues appear to have quite different ratios of GMEB-1 and GMEB-2. If there are major differences in the levels of the various isoforms among tissues, the ratios of GMEB-1 to GMEB-2 could be even more discrepant. All of the primers used in our PCR amplification of the tissue-specific cDNAs covered regions of the GMEBs that are common to the known isoforms so we cannot yet say anything about the levels of specific isoforms.

Given the major sequence differences at the C-terminal ends of the GMEB-2 isoforms, it is possible that their biological

properties may also be affected. Currently, the GMEBs have significant roles in several unrelated systems: modulation of glucocorticoid receptor induction properties (9–11,13–17,19), parvovirus replication (7) and interaction with hps27 (8). Future studies will determine if any of these activities are altered in the above isoforms.

ACKNOWLEDGEMENT

We thank Anthony Furano (NIH) for advice concerning the evolutionary relationships of the GMEBs and for critical evaluation of the manuscript.

REFERENCES

- Gross,C.T. and McGinnis,W. (1996) *EMBO J.*, **15**, 1961–1970.
- Oshima,H., Szapary,D. and Simons,S.S.,Jr (1995) *J. Biol. Chem.*, **270**, 21893–21910.
- Zeng,H., Jackson,D.A., Oshima,H. and Simons,S.S.,Jr (1998) *J. Biol. Chem.*, **273**, 17756–17762.
- Huggenvik,J.I., Michelson,R.J., Collard,M.W., Ziemba,A.J., Gurley,P. and Mowen,K.A. (1998) *Mol. Endocrinol.*, **12**, 1619–1639.
- LeBoeuf,R.D., Ban,E.M.H., Green,M.M., Stone,A.S., Propst,S.M., Blalock,J.E. and Tauber,J.D. (1998) *J. Biol. Chem.*, **273**, 361–368.
- Kaul,S., Blackford,J.A., Jr, Ogryzko,V.V., Nakatani,Y. and Simons,S.S.,Jr (1999) *Abstract No. 284*. Program and Abstracts of the 81st Annual Meeting of The Endocrine Society, Bethesda, MD, p. 499.
- Christensen,J., Cotmore,S.F. and Tattersall,P. (1999) *Mol. Cell. Biol.*, **19**, 7741–7750.
- Theriault,J.R., Charette,S.J., Lambert,H. and Landry,J. (1999) *FEBS Lett.*, **452**, 170–176.
- Mercier,L., Thompson,E.B. and Simons,S.S.,Jr (1983) *Endocrinology*, **112**, 601–609.
- Mercier,L., Miller,P.A. and Simons,S.S.,Jr (1986) *J. Steroid Biochem.*, **25**, 11–20.
- Oshima,H. and Simons,S.S.,Jr (1992) *Mol. Endocrinol.*, **6**, 416–428.
- Christensen,J., Cotmore,S.F. and Tattersall,P. (1997) *J. Virol.*, **71**, 5733–5741.
- Wasner,G., Oshima,H., Thompson,E.B. and Simons,S.S.,Jr (1988) *Mol. Endocrinol.*, **2**, 1009–1017.
- Simons,S.S.,Jr, Oshima,H. and Szapary,D. (1992) *Mol. Endocrinol.*, **6**, 995–1002.
- Szapary,D., Oshima,H. and Simons,S.S.,Jr (1992) *Endocrinology*, **130**, 3492–3502.
- Jackson,D.A., Collier,C.D., Oshima,H. and Simons,S.S.,Jr (1998) *J. Steroid Biochem. Mol. Biol.*, **66**, 79–91.
- Collier,C.D., Oshima,H. and Simons,S.S.,Jr (1996) *Mol. Endocrinol.*, **10**, 463–476.
- Zeng,H., Plisov,S.Y. and Simons,S.S.,Jr (2000) *Mol. Cell. Endocrinol.*, in press.
- Oshima,H. and Simons,S.S.,Jr (1993) *J. Biol. Chem.*, **268**, 26858–26865.
- Oshima,H. and Simons,S.S.,Jr (1992) *Endocrinology*, **130**, 2106–2112.
- Watson,J.D., Gilman,M., Witkowski,J. and Zoller,M. (1992) *Recombinant DNA*, 2nd Edn. Scientific American Books, W. H. Freeman & Co., New York, NY, p. 438.
- Li,W.-H. and Graur,D. (1991) *Fundamentals of Molecular Evolution*. Sinauer, Sunderland, MA, pp. 67–98.
- Ogawa,S., Inoue,S., Watanabe,T., Orimo,A., Hosoi,T., Ouchi,Y. and Muramatsu,M. (1998) *Nucleic Acids Res.*, **26**, 3505–3512.