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Chromosomal Organization and Transcriptional Regulation of Human *GEM* and Localization of the Human and Mouse *GEM* Loci Encoding an Inducible Ras-Like Protein

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The mitogen-induced gene, *GEM*, encodes a GTP-binding protein that belongs to a new family within the Ras superfamily. The regulated expression pattern of Gem suggests a role for this protein in cellular responses to growth stimulation. To facilitate the assessment of the possible role of *GEM* in heritable and spontaneous disease processes, the genomic organization of human *GEM* and the chromosomal localization of human and murine *GEM* have been determined. *GEM* has been localized to the long arm of human chromosome 8 (8q13–q21) between the D8S85 and *CA2* loci by genetic linkage analysis using an *Msp*I restriction fragment length polymorphism within *GEM*. No consistent somatic chromosomal alterations or heritable diseases are associated with this region. Mouse *Gem* maps to the proximal region of chromosome 4 between *Mos* and *Cga*. To gain insight into the transcriptional regulation of *GEM*, we have established the transcription initiation site of *GEM* in human T cells and defined a 5′ upstream region sufficient for mitogen-responsive, inducible transcription. © 1995 Academic Press, Inc.

INTRODUCTION

Ras proteins and their relatives function as regulatory binary switches in the cell, cycling between active GTP-bound and inactive GDP-bound states (Bourne *et al.*, 1991). Small GTPases are integral to a variety of cellular processes, including receptor-initiated signal transduction resulting in growth and/or differentiation, membrane trafficking, and cytoskeletal reorganization. Recently, a new subfamily of ras-like proteins has been described that includes Gem (Maguire *et al.*, 1994) and Rad (Reynet and Kahn, 1993). These proteins share a core sequence with the extended Ras family that is best conserved in the areas including and surrounding the motifs involved in GTP binding. However, Gem and Rad have unique structural features that distinguish them from other Ras superfamily members. Gem and Rad are larger than small GTPases as a result of extensions on the amino and carboxy sides of the Ras-like core sequence. Gem and Rad do not contain the CAAX motif responsible for lipid modification of small GTPases, despite the fact that Gem is associated with the inner face of the plasma membrane (Maguire *et al.*, 1994). Finally, Gem and Rad contain a unique G3 motif EXXG, conserved as a DXXG motif that is involved in γ phosphate binding and GTPase activity in other Ras family members (Bourne *et al.*, 1991).

In addition to having distinguishing structural characteristics, Gem is an unusual Ras family member in the regulation of its expression. Gem is not expressed in resting T cells, B cells, or fibroblasts, but is transiently expressed during G1 following the mitogenic activation of these cells. Gem is also inducible in B cells by oncogenic tyrosine kinases (Cohen *et al.*, 1994). The expression pattern of Gem suggests a role for this protein in the cellular response to growth stimulation. To evaluate the potential role of Gem in spontaneous or heritable disease processes, we have determined the genomic organization and chromosomal location of *GEM*. As a first step to investigate the mechanisms of its regulation, we have established the transcription initiation site and defined a 5′ regulatory region sufficient for inducible Gem expression.

MATERIALS AND METHODS

Genomic clone isolation and characterization. Genomic libraries were prepared as partial *Mbo*I digests of DNAs obtained from the human lung fibroblast WI-38 or mouse 129SVJ liver cloned into the λ fix II vector (Stratagene, La Jolla, CA). The libraries were screened

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. U34830 and U34831.

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with species-matched, full-length Gem cDNA probes under stringent hybridization conditions. Human Gem was subcloned as 4.0 (referred to as 270.4b)-, 3.0-, and 1.6-kb *Eco*RI fragments, listed here in a 5′ to 3′ order (Fig. 1). Murine Gem sequences that included the 5′ regulatory region were subcloned as a 6.6-kb *Bam*HI fragment, a 2.7-kb *Sma*I/*Bam*HI fragment, and a 2.3-kb *Sma*I/*Eco*RI fragment (Fig. 1). Double-stranded sequencing of the subcloned human and murine Gem using specific primers was performed. Upstream regulatory sequences (Fig. 2) have been submitted to GenBank (human, U34830; mouse, U34831).

Primer extension and RNase protection assays. The transcription initiation site was determined by primer extension and RNase protection assays. Messenger RNA was prepared from peripheral blood T lymphocytes (2×10^6/ml) that were cultured for 4 h with or without 1 μg/ml of phytohemagglutinin (Burroughs Wellcome Co.) and 20 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co.). The presence or absence of *GEM* expression was confirmed by Northern blot analyses. Poly(A)$^+$ mRNA was prepared by oligo (dT) cellulose chromatography (Stratagene) according to the manufacturer's instructions. The nonoverlapping oligomers used in primer extension analyses were primer 1, 5′-TCTCTCCCTTCTCCGTCTCGGGCC-GTCCCC-3′ [corresponding to nucleotides 76 to 47 of the lower strand of human Gem (numbering relative to GenBank Accession No. U10550)] and primer 2, 5′-GCTCGGCGGGATCGGCGTCGG-GGCGTC-3′ (nucleotides 38 to 12 of the lower strand of Gem). Primers were ^{32}P-5′-end-labeled with T4 polynucleotide kinase (Gibco) to a specific activity of $\geq 1 \times 10^8$ cpm/μg. RNA (2 μg) and primers (100,000 cpm) were denatured and then annealed at 35°C in hybridization buffer, which consisted of 0.4 *M* NaCl, 40 m*M* Pipes (pH 6.5), 1 m*M* EDTA in 80% deionized formamide. Following ethanol precipitation, the primer and template were resuspended in 100 m*M* Tris–HCl (pH 8.0), 10 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 50 m*M* KCl, 20 U of RNasin, and 100 μ*M* each of dGTP, dATP, dTTP, and dCTP. First-strand synthesis was initiated by addition of 400 U of reverse transcriptase (Superscript, Gibco), and the reaction was run at 42°C for 90 min. The extension product was ethanol precipitated, dissolved in formamide loading buffer, and resolved on a 6% polyacrylamide/7 *M* urea gel. RNase protection assays were performed using a 295-bp antisense riboprobe that spanned −137 to +106 of *GEM* (numbering relative to Genbank Accession No. U10550) in addition to 52 bp of vector sequence. RNA (5 μg) and ^{32}P-labeled probe (2×10^4 cpm) were coprecipitated, resuspended in hybridization buffer, denatured, and then annealed at 42°C for 16 h. RNase digestion was performed using 2 U RNase one (Promega) according to the manufacturer's recommendations. Samples were sized on a 6% acrylamide/7 *M* urea gel.

Transfections and CAT assays. The chloramphenicol acetyltransferase (CAT) reporter plasmid, p21.GEM-CAT, was prepared from an *Eco*RI/*Sma*I (−1715 to +27) digest of the human genomic clone 270.4b. The fragment was filled in with Klenow and blunt-end-cloned into a *Sal*I-cut, promoterless CAT reporter gene (pCAT Basic, Promega) that had been filled in with Klenow. For permanent transfections of Jurkat cells, 15 μg of p21.GEM-CAT and 1.5 μg of the pSV-2 neo plasmid were electroporated into 20×10^6 cells. Selection was performed with 1.2 mg/ml of G418 (Gibco) beginning 48 h after transfection. Cells were selected for a minimum of 4 weeks prior to being used. Inducible *GEM* promoter activity in permanently transfected Jurkat cells (5×10^6/ml) was measured after 16 h of culture in medium, ionomycin (200 n*M*), PMA (20 ng/ml), or ionomycin (200 n*M*) plus PMA (20 ng/ml). CAT activity was quantified in cell lysates using a fluorochrome assay (Neumann *et al.*, 1987).

Human GEM mapping. Southern blots of genomic DNA restriction digests on positively charged nylon membranes were prepared after

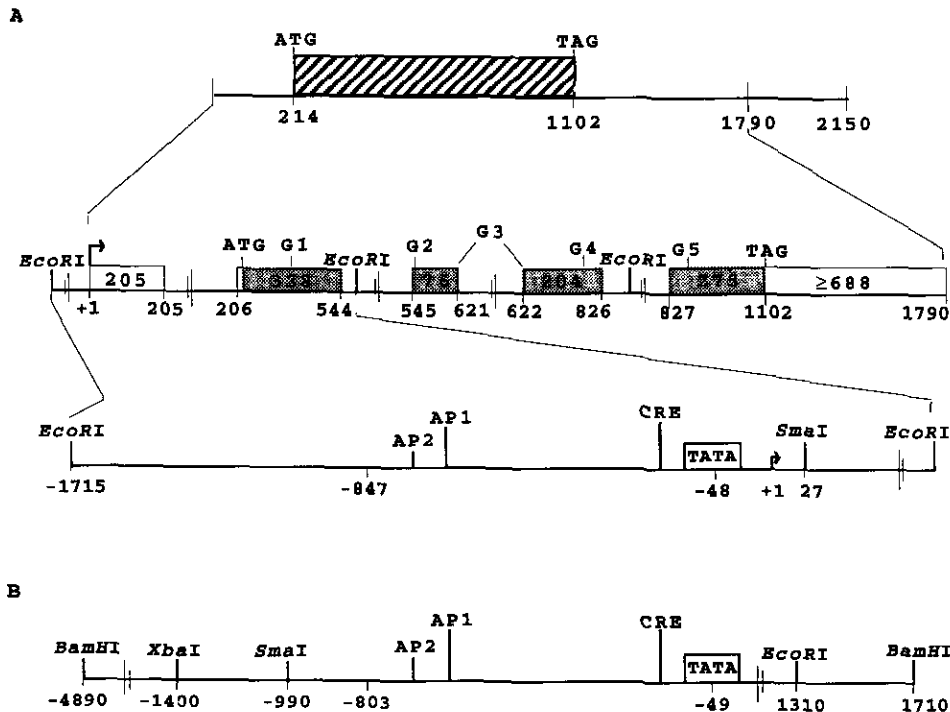


FIG. 1. Genomic structure of human *GEM* and of the murine *Gem* upstream region. (**A**) The cDNA structure and features of the human *GEM* genomic clones encompassing the protein coding regions and upstream regulatory sequences. Protein coding regions are shown as shaded boxes and untranslated regions as open boxes. The numbers within the boxes represent the nucleotide length of exons; the numbers below the upper genomic diagram represent the nucleotide number of the exon–intron boundaries relative to the cDNA sequence (Accession No. U10550). The approximate locations of the GTP-binding domain concensus sequences, G1 to G5, are shown. In the lower genomic diagram of clone 270.4b, numbering is relative to the transcription initiation site (+1) that is indicated by an arrow (see Fig. 2). Included are the positions of potential regulatory *cis*-acting elements. *Eco*RI sites used in subcloning are indicated. (**B**) Mouse genomic clone 18.2 encoding upstream regulatory sequences. Numbering has been assigned by similarity to the human clone, where +1 is the start of transcription. Double vertical lines indicate introns or other noncoding sequence not drawn to scale.

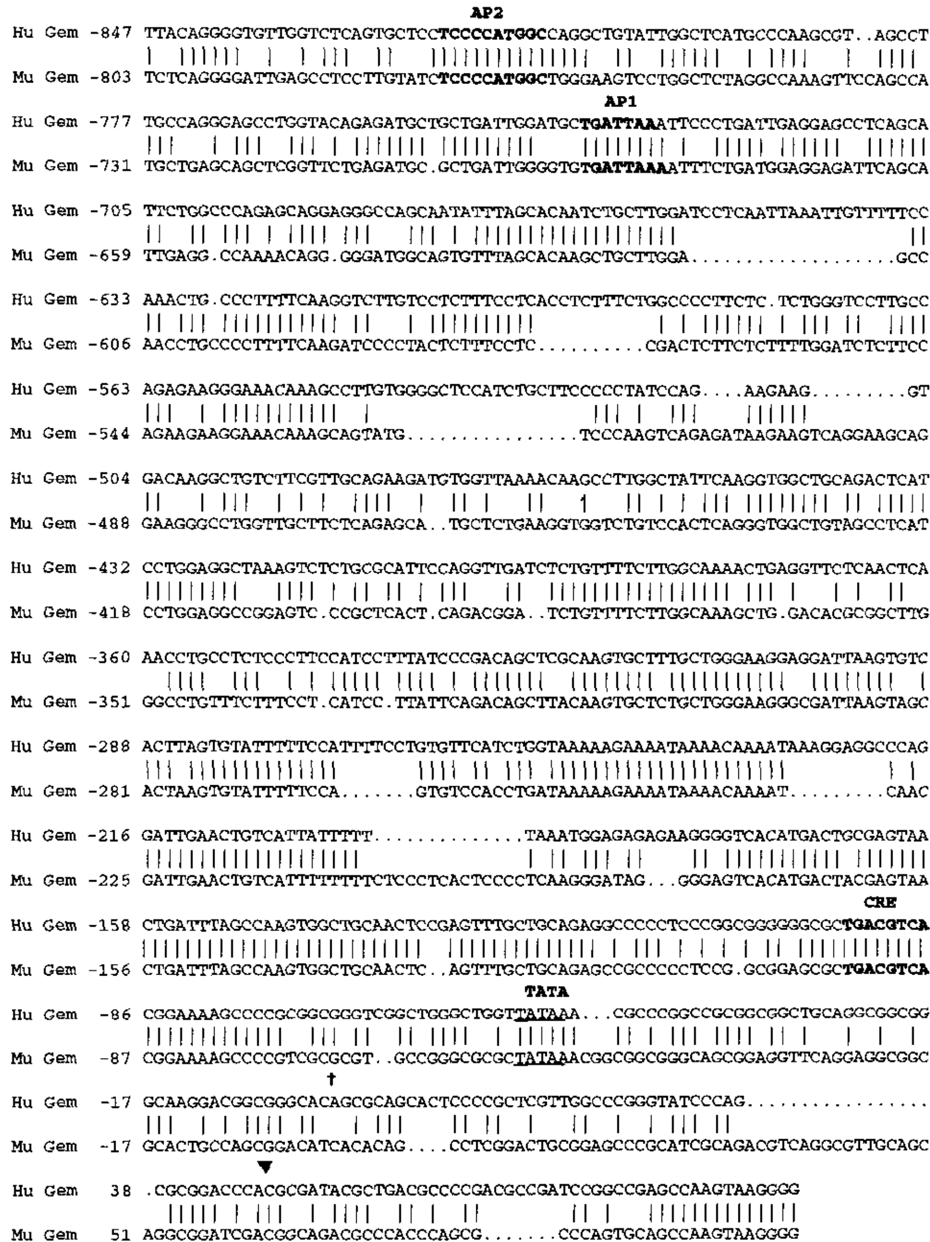


FIG. 2. Nucleotide sequences of the human and murine *GEM* promoters. Upstream sequences derived from human (Hu) genomic clone 270.4b (GenBank Accession No. U34830) and mouse (Mu) genomic clone 18.2 (GenBank Accession No. U34831) are shown. The transcription initiation site (nt +1) of the human promoter is indicated by a cross. The arrowhead (nt 49) signifies the cDNA start site of the published sequence. The numbering of clone 18.2 has been assigned by similarity to the human clone, where +1 is the start of transcription. Potential regulatory *cis*-acting elements are displayed in boldface. The TATA box is underlined. Dashes have been inserted using the BESTFIT program to maximize sequence alignment.

(0.7%) agarose gel electrophoresis and hybridized at high stringency with ^{32}P-labeled probes (Olson *et al.,* 1991) under conditions allowing no more than a 10% divergence of hybridizing sequences. Blots were reused after the removal of probe with alkali and neutralization. Isolation and characterization of a panel of human/rodent somatic cell hybrids have been described (McBride *et al.,* 1982; Tomfohrde *et al.,* 1992). To evaluate DNA restriction fragment length polymorphisms, DNA from 10 unrelated normal individuals was separately digested with 12 different restriction enzymes (*Eco*RI, *Hin*dIII, *Bam*HI, *Xba*I, *Sac*I, *Taq*I, *Msp*I, *Pvu*II, *Pst*I, *Bgl*II, *Eco*RV, and *Kpn*I) and examined for restriction length polymorphisms (RFLPs) by Southern blotting with a 2.1-kb *GEM* cDNA probe after agarose gel electrophoresis.

Mouse Gem mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *M. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *Gem* locus, as detailed. Southern blot analysis was performed as described (Jenkins *et al.,* 1982). All blots were prepared with Hybond-N$^+$ membrane (Amersham). A 2.3-kb *Sma*I/*Eco*RI fragment (nucleotides −990 to 1310 of the mouse genomic clone) was labeled with [α-^{32}P]dCTP using a random priming labeling kit (Amersham). Washing was performed to a final stringency of 0.5× SSCP, 0.1% SDS, 65°C. A 10-kb fragment was detected in *Hin*dIII-digested C57BL/6J DNA, and a 18-kb fragment was detected in *Hin*dIII-digested *M. spretus* DNA. The presence or absence of the 18-kb *M. spretus*-specific fragment was followed in backcross mice.

The probes and RFLPs for the loci linked to *Gem*, including Moloney sarcoma oncogene (*Mos*), glycoprotein hormone, α subunit (*Cga;*

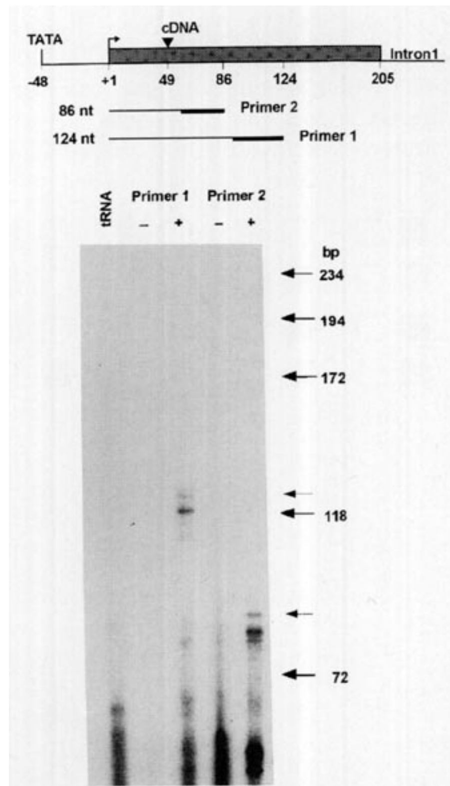


FIG. 3. The transcription initiation site of *GEM*. End-labeled, antisense oligonucleotides were used in primer extension assays. Primers 1 and 2 spanned the reverse complements of nts 47 to 76 and 12 to 38, respectively (numbering relative to GenBank Accession No. U10550). Primers 1 (lanes **1–3**), and 2 (lanes **4** and **5**) were annealed to tRNA (lane **1**) or to Poly(A)$^+$ mRNA (lanes **2–5**) derived from human peripheral blood T lymphocytes cultured for 4 h in the presence (+) or absence (−) of PHA (1 μg/ml) plus PMA (20 ng/ml). Thick arrows indicate size markers (bps) from *Hae*III-cut PhiX174. Thin arrows indicate size of longest extension products using primer 1 (124 bp) or primer 2 (86 bp).

formerly *Tsha*), and ciliary neurotrophic factor receptor α (*Cntfr*), have been reported (Ceci *et al.*, 1989; Valenzuela *et al.*, 1995) previously. Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

To determine the structure of *GEM,* human genomic clones were isolated, the locus was mapped relative to the cDNA structure, and the intron–exon boundaries were established with nucleotide sequencing. The *GEM* gene, including 1715 nucleotides 5′ of the transcription initiation site and inclusive of the cDNA through nucleotide 1790, spanned a region of 8.6 kb and consisted of five exons. The first exon of *GEM* is noncoding, and the remaining four encode the Gem protein (Fig. 1). The fifth exon additionally contains 3′-untranslated sequence. The short structural motifs (G1 through G5) that mediate guanine nucleotide binding (Bourne *et al.*, 1991) are distributed among the four coding exons. The G3 motif that is unique in Gem and Rad relative to other small GTPases is encoded noncontiguously in exons 3 and 4. Despite the fact that Ki-ras is 29% similar to Gem (Maguire *et al.*, 1994), the genomic loci for the two genes show no common organization of intron–exon boundaries (McGrath *et al.*, 1983; Shimizu *et al.*, 1983).

To facilitate investigations into the regulation of *GEM* expression, the sequences of the regions upstream of the cDNA start sites were determined for human and murine clones (Figs. 1 and 2). Allowing for gaps, 75% identity was observed in the 847 upstream basepairs that were analyzed. Human and mouse *GEM* contain conserved TATA elements, consensus cAMP response elements (CRE), and AP1 and AP2 sites. The TATA box and approximately 150 bp of 5′-flanking sequence, including the CRE site, are highly homologous in the two species. There is relatively little homology in the human 5′-untranslated sequence compared to that in the aligned mouse sequence (Fig. 2).

The transcription initiation site of *GEM* in mitogen-activated human peripheral blood T cells was identified by primer extension assays. Results were in good agreement using two nonoverlapping oligonucleotides that mapped the start site to 48 bp downstream of the TATA box (Fig. 3). A similar transcription initiation site was predicted by RNAse protection assays (data not shown). No specific products were observed in primer extension assays using RNA derived from resting peripheral blood T cells that are transcriptionally silent for *GEM*. The 5′ end of a *GEM* cDNA isolated from murine B cells (Cohen *et al.*, 1994) is distinct from the sequences identified here, implying the existence of more than one transcription initiation site.

The functional activity of the *GEM* 5′ regulatory region was confirmed by its effect on CAT reporter expression. Nucleotides −1715 to +27 of human clone 270.4b were fused to a promoterless CAT reporter gene (p21.Gem-CAT). Transient transfections of a variety of

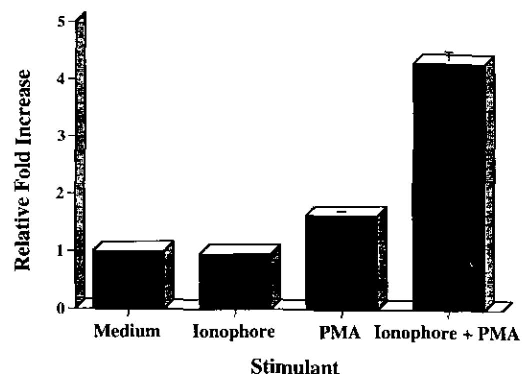


FIG. 4. Inducible *GEM* promoter activity. Jurkat cells permanently transfected with p21.Gem-CAT (2 × 10^6/ml) were either untreated or treated with 0.2 μ*M* ionomycin, PMA (20 ng/ml), or ionomycin (0.2 μ*M*) plus PMA (20 ng/ml) for 16 h prior to harvesting and determination of CAT activity. Data reflect the fold increase in CAT relative to the medium control, which was assigned a value of 1. Results are derived from three separate transfections.

TABLE 1

Two-Point Lod Scores for GEM Versus Other Loci on Chromosome 8

Loci[a]	θ^b	Z^c	Location
1. D8S87	0.292	3.643	8p12
2. PLAT	0.683	0.449	8q12–q11.2
3. CA3	0.00	6.085	8q13–q22
4. CA2	0.104	9.391	8q13–q22
5. D8S84	0.148	12.452	8q13–q21.2
6. D8S2	0.100	10.385	8
7. D8S85	0.132	21.274	8q23–qter
8. MYC	0.500	0.00	8q24.12–q24.13

[a] The following loci were typed using short tandem repeat polymorphisms: D8S87 (Tomfohrde *et al.*, 1992); *PLAT* (Tomfohrde *et al.*, 1992); D8S84 (Tomfohrde *et al.*, 1992); D8S85 (Tomfohrde *et al.*, 1992); and *MYC* (Group, 1992). Probe–enzyme combinations for the other loci were D8S2 (I82B, *Taq*I) (Group, 1992), *CA2* (H25-3.8, *Taq*I) (Bailey *et al.*, 1985), and *CA3* (pCA15, *Taq*I) (Lloyd *et al.*, 1986).

[b] The most likely recombination fractions (sex average value) between *GEM* and these loci are shown.

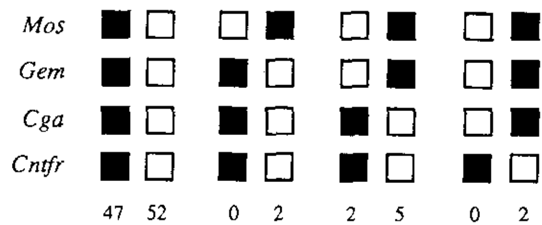
[c] Lod scores at θ_{max} assuming sex average recombination fraction.

cell lines (NTERA-2, NIH 3T3, Jurkat, EL-4, and U937) revealed a 25- to 75-fold increase in promoter activity with the p21.Gem-CAT construct relative to the CAT vector alone (data not shown). Further inducible transcription from the *GEM* 5′ regulatory region was not observed in transient transfection assays.

We reasoned that the extrachromosomal location of *GEM* 5′ regulatory sequences, as occurs in transient assays, was responsible for a lack of normal regulation. Therefore, a polyclonal Jurkat cell line, permanently transfected with p21.Gem-CAT, was prepared. Activation of transfected Jurkat cells with PMA and calcium ionophore, which has been shown to mimic the signals generated by triggering the T-cell receptor, resulted in a fourfold increase in CAT reporter expression (Fig. 4). Transcriptional induction of *GEM* required two signals, as neither calcium ionophore nor PMA alone was sufficient.

The *GEM* locus was localized to human chromosome 8 by Southern analysis of a panel of *Eco*RI-digested human/rodent somatic cell hybrid DNAs using a full-length 2.1-kb *GEM* cDNA as a probe. The gene segregated concordantly (100%) with chromosome 8 and discordantly (equal to or greater than 25%) with all other chromosomes in the panel of hybrids (data not shown). Genetic linkage analyses of CEPH families were used to refine further the position of *GEM*. Southern blots of restriction digests (*Eco*RI, *Hin*dIII, *Bam*HI, *Xba*I, *Sac*I, *Taq*I, *Msp*I, *Pvu*II, *Bgl*II, *Eco*RV, and *Kpn*I) of DNAs from 10 unrelated individuals revealed RFLPs for *Msp*I and *Pvu*II. *Msp*I RFLPs among the 80 CEPH parents consisted of three alleles with frequencies of A1:A2:A3 = 0.67:0.01:0.32; heterozygosity, 0.46; and PIC = 0.36. There were two *Pvu*II RFLPs, each with two alleles and frequencies of A1:A2 = 0.35:0.65 and B1:B2 = 0.90:0.10. Analysis of the CEPH parents revealed complete linkage disequilibrium between the *Msp*I and the *Pvu*II RFLPs. Therefore, the *Msp*I RFLP was used for genotyping all members of the 30 informative families in which one or both parents were heterozygous at the GEM loci.

Linkage analyses with other loci in the CEPH data-



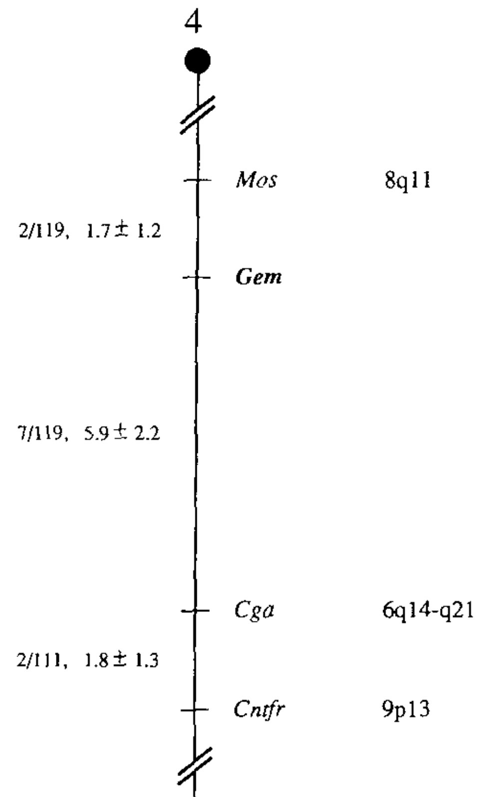


FIG. 5. Chromosomal location of *Gem* in the mouse genome. The locus was mapped by interspecific backcross analysis. (**Top**) The segregation patterns of *Gem* and the flanking genes. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F_1 parent. The black boxes represent the presence of a C57BL/6J allele, and the white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. (**Bottom**) A partial chromosome 4 linkage map showing the location of *Gem* in relation to linked genes. The number of recombinant N_2 animals over the total number of N_2 animals typed plus the recombination frequencies, expressed as genetic distance in centimorgans (± one standard error), is shown to the left for each pair of loci. The positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from the GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

TABLE 2

Multipoint Linkage Analysis of *GEM* with Other Loci on Chromosome 8

Odds:		(5.9×10^7)		(1.1×10^5)		(44.5)		(6.3×10^{11})	
θ_{male}		*0.043*		*0.086*		*0.00*		*0.126*	
	D8S85	—	*GEM*	—	*CA2*	—	D8S84	—	D8S87
θ_{female}		0.222		0.121		0.179		0.564	
θ_{sexave}		0.122		0.073		0.108		0.270	

Note. Five loci were ordered using the CILINK program (see Results). The most likely recombination fractions between loci and the odds against reversing the order of adjacent loci (in parentheses) are shown.

base were performed. The loci in Table 1 are ordered from short arm to long arm based upon published extensive multipoint linkage analyses (Wood *et al.*, 1993). These loci span a distance of about 80 cM (sex average) on the CEPH consortium map, representing about 34% of the entire genetic distance encompassed by chromosome 8 and spanning the region from about 8p12 to q24. The highest lod scores were with D8S85 (LOD = 21.274) and D8S84 (LOD = 12.452).

GEM was ordered within this group of loci by multipoint linkage analysis (Table 2). *GEM*, D8S84, D8S85, D8S87, and *CA2* were ordered using the program CILINK while considering all 60 possible orders. The most likely order is shown in Table 2, although *CA2* and D8S84 could not be ordered with high probability. This analysis places *GEM* between D8S85 and *CA2* within the region 8q13–q22. The order of published loci places D8S84 closer than *CA2* to D8S85, although *CA2* and D8S84 are very closely linked (4.4 cM, sex average). The mouse chromosomal location of the *Gem* locus was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1900 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative RFLPs using a 2.3-kb *Sma*I/*Eco*RI fragment of the mouse genomic clone as a probe (Fig. 1B). The mapping results indicated that *Gem* is located in the proximal region of mouse chromosome 4 linked to *Mos*, *Cga*, and *Cntfr* (Fig. 5). Although 110 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 5), up to 196 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the recombination frequencies between the loci are shown in Fig. 5.

DISCUSSION

GEM has been localized to human chromosome 8 between bands q13 and q21 between *CA2* and D8S85, and the murine homologue has been localized in the proximal region of chromosome 4 between *Mos* and *Cga*. The mapping in the two species is consistent, as the proximal 10.5 cM of murine chromosome 4 are homologous with human chromosome 8 (Abbott *et al.*, 1994). At this time, there are no specific human chromosomal rearrangements, deletions, or heritable disease loci that have been mapped between the markers that flank *GEM*, although cytogenetically cryptic mutations are possible. In addition, we have compared our chromosome 4 interspecific map with a composite mouse linkage map that reports the map location of many uncloned mouse mutations provided by the Mouse Genome Database (The Jackson Laboratory, Bar Harbor, ME). Although several uncloned mouse mutations appear to lie in its vicinity, none of these mutations has a phenotype consistent with what might be predicted for a mutation in *Gem*.

We have characterized a 1.0-kb 5′ upstream region of *GEM* that contains a TATA box appropriately situated relative to the transcription initiation site in addition to a number of potential transcription factor binding sites. Of note is a CRE site at −94 to −87 embedded within a region highly conserved between mouse and human that may prove to be important in regulated expression. Using polyclonal, permanently transfected Jurkat cells, we have shown that elements within the 1.0-kb upstream region are sufficient for inducibility by calcium ionophore and PMA. Interestingly, two signals appear to be required for *GEM* transcriptional induction in Jurkat cells (see Fig. 4), as neither PMA nor calcium ionophore alone induces reporter gene activity. A requirement for two signals in the induction of transcription could reflect a requirement for two signals leading to activation of a single regulatory site or, alternatively, may reflect a requirement for activation through several nonredundant sites in the 5′ upstream region.

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