Characterization of the murine phosphatidylethanolamine *N*-methyltransferase-2 gene[1]

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Abstract Phosphatidylethanolamine *N*-methyltransferase (PEMT) catalyzes the conversion of phosphatidylethanolamine to phosphatidylcholine in the mammalian liver via three sequential methylations. In the present studies, we cloned and characterized the murine gene for PEMT2, the isoform of the enzyme that localizes to the mitochondria-associated membrane. The structure of the gene was determined by analysis of two λ and three P1 genomic clones, and compared to the known rat PEMT2 cDNA sequence. Southern blotting of mouse genomic DNA indicated that PEMT2 is a single-copy gene. The gene spans at least 35 kb, with seven exons and six introns. Two transcription start sites, 139 and 148 base pairs upstream of the translation start site, were detected by primer extension and reverse transcriptase–polymerase chain reaction. These experiments indicated that the PEMT2 gene is transcribed from a single promoter. Finally, the PEMT2 gene was localized to mouse chromosome 11 by interspecific backcrossing. These experiments represent the first cloning and characterization of a full-length mammalian gene involved in phospholipid biosynthesis.—**Walkey, C. J., Z. Cui, L. B. Agellon, and D. E. Vance.** Characterization of the murine phosphatidylethanolamine *N*-methyltransferase-2 gene. *J. Lipid Res.* 1996. **37:** 2341–2350.

Supplementary key words phosphatidylcholine • gene structure • primer extension • chromosomal localization

Phosphatidylcholine (PC) is the most abundant phospholipid in eukaryotic cells, functioning both as a structural component of membranes and as a source of second messengers. PC synthesis via CDP-choline occurs in all eukaryotic cells. An alternative pathway for PC synthesis, the conversion of phosphatidylethanolamine (PE) to PC, is largely confined to the liver (1). Three sequential methylation steps are catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT), using *S*-adenosylmethionine as the methyl group donor. At least two PE methyltransferases exist in the liver: PEMT1, which is localized primarily to the endoplasmic reticulum, and PEMT2, which is present solely on the mitochondria-associated membrane (2). The reasons for hepatocytes having two distinct isoforms of PEMT are unclear. A full-length cDNA for PEMT2 has been isolated from rat liver (2).

Recent investigations in our laboratory suggest a strong inverse correlation between PEMT2 expression and liver cell growth. During embryogenesis, when hepatocytes grow and divide rapidly, PEMT activity is low (3). After birth, the rate of hepatocyte division slows (4), while PEMT activity rises considerably. This growth stage-specific regulation appears to occur at the level of gene expression (Z. Cui and D. E. Vance, unpublished observations). Rapidly dividing hepatoma-derived cell lines (McA-RH7777 and HepG2) have almost undetectable PEMT activity. Expression of the rat liver PEMT2 greatly slows the growth rate of McA-RH7777 cells, but does not alter the growth of Chinese hamster ovary cells in culture (5). Chemically induced liver tumors contain lower levels of PE methylation activity than normal liver, and no PEMT2 protein (L. Tessitore, Z. Cui and D. E. Vance, unpublished observation). As well, PEMT2 expression is greatly decreased during non-neoplastic liver growth. The growth inhibitory effects of PEMT2 appear to be directly related to the enzymatic conversion of PE to PC.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

[1]The nucleotide sequences reported in this paper have been submitted to the Genbank™/EMBL Data Bank with the accession numbers: exon 1, U25045; exon 2, U25046; exon 3, U25047; exon 4, U25048; exon 5, U25049; exon 6, U25050; exon 7, U25051. The chromosome localization of the *Pempt2* locus has been accessioned to the Mouse Genome Database (MGD) with the accession number: MGD-MRK-25966.

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Using the rat PEMT2 cDNA as a probe, we have cloned and characterized the full-length mouse gene for PEMT2.

MATERIALS AND METHODS

Isolation and characterization of genomic clones

A 129/Sv strain mouse liver genomic DNA library (gift from Dr. Jim Stone, University of Alberta) was screened with random primer-labeled rat PEMT2 cDNA. Two positive clones, named 3 and 11, were isolated from an initial screen of approximately 2×10^7 plaques, and characterized by restriction mapping, Southern blotting, and partial sequencing. A P1 library (Genome Systems Inc.) containing inserts up to 110 kb was screened using polymerase chain reaction (PCR) primer pairs based on sequences from clones 3 and 11[Exon1for: 5′-AGAACTTAGGAGTCAAGCCTCC-3′; Exon1rev: 5′-CTCTTCACACCTGAGACATGG-3′; Exon5for: 5′-TCAAGGAGTCCAGAGTGACC-3′; Exon5rev: 5′-CTTGGATCCGGATACCTAGG-3′]. Three P1 clones were isolated. One of the P1 clones, 2614, spanned the entire PEMT2 gene, according to Southern blotting and partial sequencing.

Sequencing was performed by the DNA Core facility, University of Alberta, using an automated sequencer (Applied Biosystems Inc./Perkin-Elmer model 373A). The results were analyzed using the Intelligenetics Geneworks program. Exon sequences were determined from at least two different clones, and intron/exon boundaries were defined by comparison to the rat PEMT2 cDNA sequence. DNA preparations, restriction digests, and ligations were performed by standard methods. P1 DNA was isolated using a Qiagen DNA preparation kit following the manufacturer's protocol.

Southern blotting

One μg of clone DNA and 10 μg of mouse genomic DNA were digested with various restriction enzymes. After restriction digestions, DNA fragments were electrophoresed through 1.0% agarose, transferred to Hybond N+ (Amersham) nylon membranes following the manufacturer's instructions, and probed with the rat PEMT2 cDNA labeled by random priming. The hybridizations were performed at 42°C, in 50% formamide containing 100 mg/ml salmon sperm DNA. The membranes were washed under stringent conditions of 0.1 × SSC (15 mM NaCl, 1.5 mM Na citrate, pH 7.0), 0.1% SDS at 52°C, and exposed to X-ray film.

RNA primer extension assay

Total RNA was isolated from 8-week-old male 129/J strain mouse livers essentially as described by Chomczynski and Sacchi (6). One pmol of a 30-base oligonucleotide primer, rev-1: 5′-CTGTGGGGTCCACGTAACC CAGCAGCCAGC-3′ was labeled with [γ-^{32}P]ATP at its 5′ end by T4 polynucleotide kinase (Life Technologies Inc.), then hybridized to 40 μg of total mouse liver RNA at 30°C for 16 h (7). Extension reactions were catalyzed using SuperScript reverse transcriptase (Life Technologies Inc.). The reaction products were separated by electrophoresis (5% polyacrylamide in 8 M urea), and visualized by autoradiography. The rat PEMT2 cDNA was sequenced with rev-1 as a primer using Sequenase 2.0 (Amersham) and following the manufacturer's instructions, and electrophoresed alongside the extension products as a size marker. By comparing the position of the extension product bands to the sequencing ladder alongside, the length of the extension products, and hence the transcription start sites, were determined.

Reverse transcriptase-PCR

Primer extension products were eluted from the polyacrylamide gel by incubation for 48 h at 37°C in 0.5 M ammonium acetate, 1 mM EDTA, pH 8.0, and precipitated with ethanol. The primer extension products were then used as templates for PCR. Blank spots on the gel were also eluted as negative controls. The PCR reactions were performed in 50-μL volumes containing 0.25 mM dNTPs and 1.5 mM $MgCl_2$, for 30 cycles of 95°C for 30 s, 54°C for 30 s, and 74°C for 30 s, plus a final extension step of 2 min. Primers were rev-2, 5′-CAGAACCAGAAG GAAATGG-3′; for-1, 5′-TGGTAGAACTGCTATGTG CCG-3′; for-2, 5′-TTCCCTAACTACAGAACATTGC-3′. The PCR products were analyzed on a 1% agarose gel.

PEMT2 mRNA and protein analysis

Poly A-enriched RNA was isolated from adult male rat livers using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech), according to manufacturer's instructions. PEMT2 mRNA was determined by Northern blot analysis. Briefly, 2-μg RNA samples were electrophoresed and blotted onto a nylon membrane according to standard techniques (7). The blots were probed with rat PEMT2 cDNA labeled by random priming, under the same hybridization conditions as for Southern blotting, then washed under stringent conditions (0.1 × SSC, 55°C) prior to autoradiography.

Samples of rat and mouse adult male liver homogenates containing 50 μg total protein were analyzed for the presence of PEMT2 protein by SDS-PAGE immunoblotting, exactly as previously described (2).

Chromosome mapping

Interspecific backcross progeny were generated by mating (C57BL/6JEi × *M. spretus*) F_1 females with *M. spretus* males (8) as described. Genomic DNA was digested with a selection of restriction enzymes, and probed with a 1.9 kb *Pst1* restriction fragment of clone 11 containing exon 5 radiolabeled by random priming. A single 6.6 kb band was detected in C57BL/6JEi genomic DNA digested with *Bgl2,* while two bands of 3.2 and 4.0 kb were found in *M. spretus* genomic DNA digested with *Bgl2.* This restriction fragment length polymorphism (RFLP) was followed in 94 N_2 offspring, and the segregation distribution pattern determined. Comparison to previously mapped loci and calculations of map distances were performed by the BC Panel Map Service at the Jackson Laboratory.

RESULTS

Cloning and characterization of the mouse PEMT2 gene

Full-length rat PEMT2 cDNA was used to screen a library of 129/Sv strain male mouse liver genomic DNA, cloned in the bacteriophage vector λDASH2. Two 15 kb clones, designated 3 and 11, were isolated and subcloned into pBluescriptII- SK+Stratogene. Southern blots revealed that clone 3 hybridized to the 5′ end of the PEMT2 cDNA, while clone 11 hybridized to the middle portion of the cDNA. The recognition sites of several restriction enzymes were mapped on both clones, using standard single and double digestion techniques. Using this restriction map, the fragments containing exons were identified by probing a Southern blot with rat PEMT2 cDNA, and sequenced. Consequently the number, size, and location of exons in the PEMT2 gene were determined. Clone 3 contained two exons corresponding to the 5′ end of the PEMT2 cDNA as predicted (**Fig. 1**). Clone 11 contained 3 adjacent exons. As neither clone contained the 3′ end exons, despite spanning 30 kb in total, a commercial 129/J mouse genomic DNA library, packaged in P1 phage-derived plasmid, was screened by PCR, using primers specific for exons I and V. One clone, 2614, that spanned the full length of the gene was isolated. Two additional clones, 2615 and 2616, spanned the 3′ end only (Fig. 1). The P1 clones were subcloned and analyzed in the same manner as for the λ clones.

It appears that the murine PEMT2 gene is at least 35

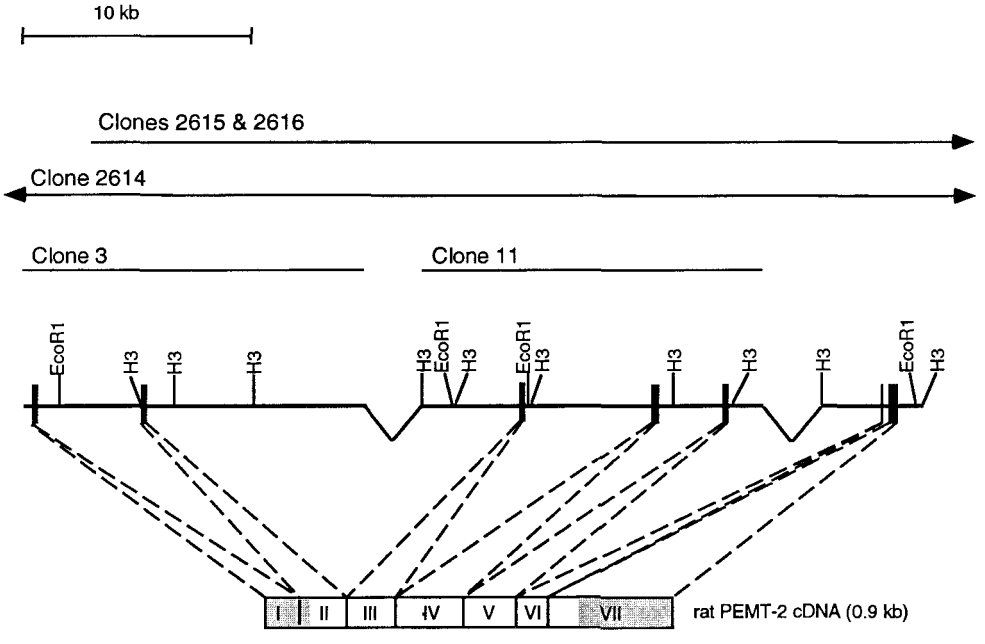

Fig. 1. Schematic representation of the genomic clones encompassing the mouse PEMT2 gene (top) and intron/exon organization of the gene (bottom). The position and size of the λ and P1 clones encompassing the 7 exons of the murine PEMT2 gene are shown as solid lines. Arrows indicate that the ends of the clones remain undetermined. Exons are represented by vertical solid boxes joined by solid lines representing introns. Divots in the introns indicate regions of unknown size. The relationship between the gene and the rat PEMT2 cDNA is shown below, depicting the relative sizes and organization of the exons. The shaded and unshaded areas represent untranslated and translated regions respectively.

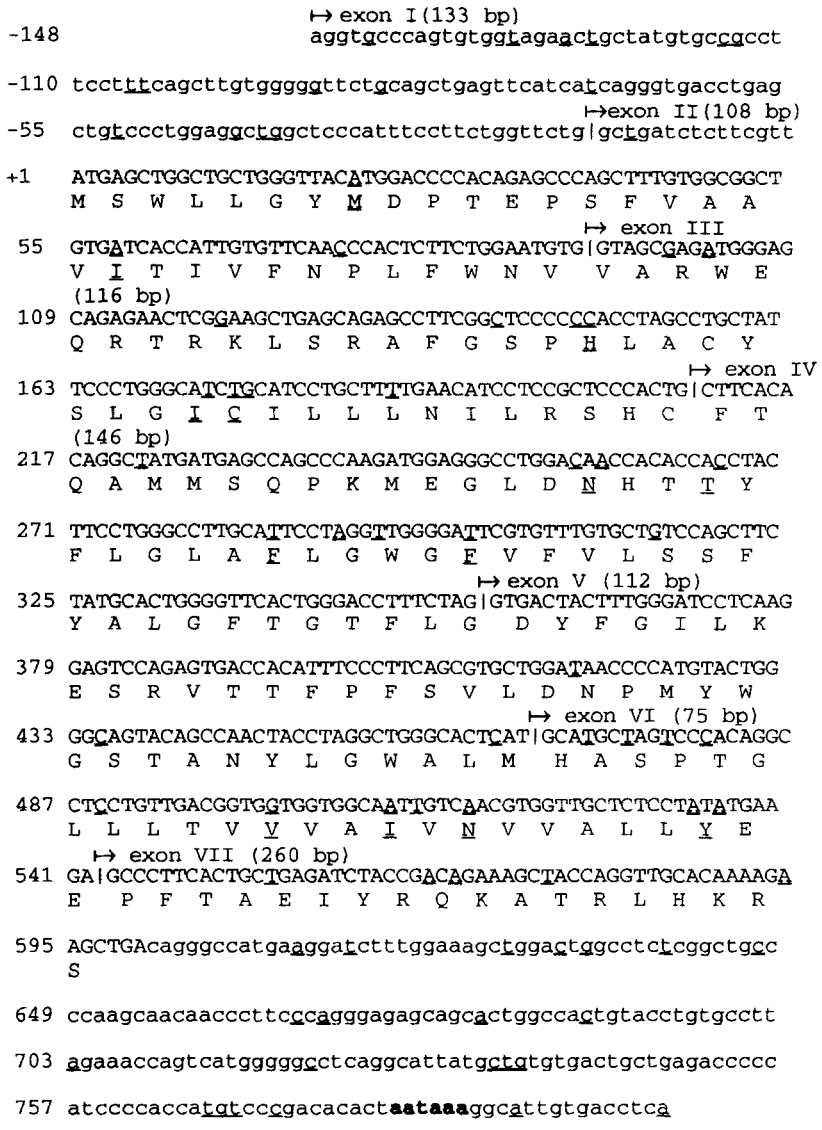

Fig. 2. Putative mouse PEMT2 cDNA based on exon sequences. The sequences of all seven exons have been combined as in a fully spliced mouse PEMT2 mRNA. Junctions between exons are shown as vertical lines. The start of exon I is based on the primer extension results discussed in the text. Non-coding nucleotides are in lower case; coding nucleotides are in upper case; +1 indicates the first nucleotide of the translation start codon. Translation products of exons are shown below the nucleotide sequences. Nucleotide and amino acid changes from the rat PEMT2 cDNA sequence are underlined. The boldface type indicates the polyadenylation signal.

kb long, with seven exons. Each exon was sequenced from at least two different clones (Fig. 1 and **Fig. 2**). These exon sequences from mouse are 92.8% identical to the rat PEMT2 cDNA sequence at the nucleotide level, and 93.5% identical at the amino acid level. The intron/exon boundaries in the genomic clones were deduced by sequence alignment to the rat PEMT2 cDNA (**Table 1**). The sequences of these boundary regions, especially the conserved GT and AG dinucleotides, match the consensus for pre-mRNA splicing recognition sites (9).

Genomic DNA from a 129/J mouse and clone 2614 DNA was digested with four different restriction enzymes and probed with rat PEMT2 cDNA in a Southern blot (**Fig. 3**). Identical bands were detected in both the genomic and the cloned DNA, indicating that clone 2614 accurately reflects the PEMT2 gene structure. The lack of additional bands of equal intensity in the genomic blot also indicates that mouse PEMT2 exists as a single-copy gene without pseudogenes. In other words, there is no complete or partial duplication of the PEMT2 gene elsewhere in the mouse genome. Additional faint bands visible in the genomic blot are mostly likely due to allelic variation at the PEMT2 locus in the genomic DNA from the 129/J mouse, or segments of genomic DNA weakly homologous to PEMT2.

Mapping of the transcription initiation sites

The transcription start site(s) of the PEMT2 gene were determined by primer extension analysis, using the 30-base oligonucleotide rev-1, complementary to exon 2 of mouse PEMT2 mRNA. The extended prod-

TABLE 1. Exon/intron organization of the mouse PEMT2 gene

Intron	5′ Splice Donor	Intron Length *kb*	3′ Splice Acceptor	Codon Phase	Amino Acid at Splice Site
1	**GTTCTG**GTAGGT	5.0	TTGCAG**GCTGAT**	—	—
2	**AATGTG**GTGAGT	>13.0	ATGCAG**GTAGCG**	O	32–Val/Val
3	**CCACTG**GTAAGC	5.7	CCACAG**CTTCAC**	II	70–Cys
4	**TTCTAG**GTAAGA	2.9	CCACAG**GTGACT**	I	119–Gly
5	**ACTCAT**GTGAGT	>4.4	CTGCAG**GCATGC**	II	156–Met
6	**TGAAGA**GTGAGT	0.3	CCACAG**GCCCTT**	II	181–Glu

Nucleotide sequences were determined by sequencing exon containing subclones of the λ and P1 genomic clones (see Methods). Exon sequences are **boldface.** The invariant GT (5′) and AG (3′) dinucleotides at the ends of the introns are underlined. The position of each splice site relative to a codon is shown as the codon phase. O indicates a splice site between codons, I indicates a splice site after the first nucleotide of a codon, and II indicates a splice site after the second nucleotide of a codon. Amino acids are numbered from the initiator methionine.

ucts were sized by comparison to a DNA sequencing ladder. The results suggested two transcription start sites: a major one 139 bp upstream of the initiator methionine codon, and a second minor start site 148 bp upstream (**Fig. 4a**). The primer extension products were eluted from the sequencing gel and used as templates for PCR amplifications. A primer 3′ to the putative start sites (for-1). in conjunction with another PEMT2-specific primer (rev-2), was able to amplify each extension product [Figs. 4b(i) and 4c], confirming the extension products as being PEMT2 cDNAs rather than the product of nonspecific hybridization of the original 30mer. However, a primer specific to the region beyond the putative start sites (for-2) failed to amplify the extension products [Fig. 4b(ii)]. This result confirms the location of the PEMT2 gene transcription start sites. Both transcription start sites have the same 4 base motif, CCAG. In both cases, the first nucleotide of the transcribed RNA is A, a purine. There does not appear to be a consensus TATA box (TATAA) in the region 30 bp upstream (10, 11). A putative mouse PEMT2 cDNA based on the exon sequencing and primer extension data is shown in Fig. 2. This construct predicts PEMT2 transcripts of approximately 900 bp, while the largest open reading frame predicts a 199-amino acid PEMT2 protein with a molecular weight of 22.5 kDa.

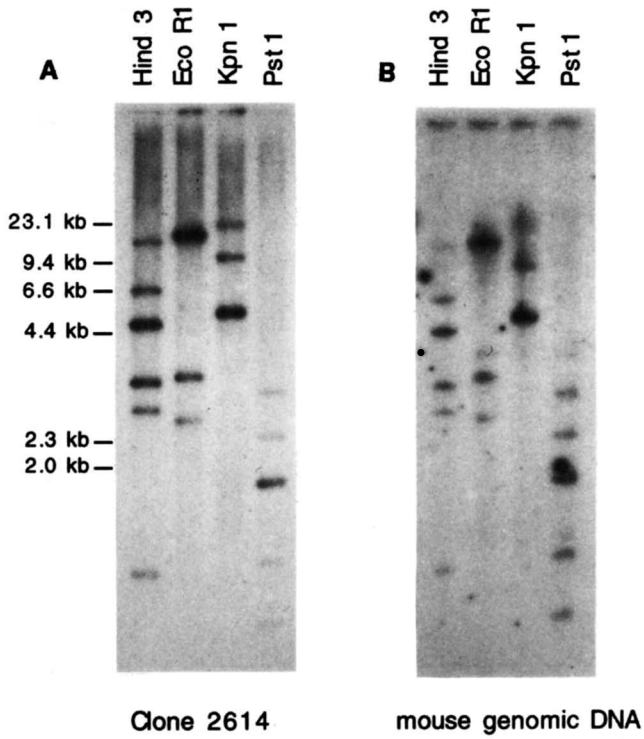

Fig. 3. Southern blotting of clone 2614 and mouse genomic DNA probed with rat PEMT2 cDNA. One μg of DNA from clone 2614 and 10 μg of 129/J strain mouse genomic DNA were digested with the restriction enzymes indicated at the top of the figure, electrophoresed through a 1% agarose gel, alkali blotted and probed with rat PEMT2 cDNA, as described in Materials and Methods. The numbers on the left side indicate the positions of DNA size markers.

Gene product analysis

To confirm the gene structure presented above, Northern and Western blot analyses were performed on mouse liver samples. Probing mouse total RNA with rat PEMT2 cDNA revealed one band, indicating a single transcript of approximately 900 bp, with a high level of identity to the rat PEMT2 transcript (**Fig. 5a**). The size of this transcript matches that predicted above. The nine base pair difference in transcript length due to the two different transcription start sites is too small to be resolved into two distinct bands.

Mouse and rat liver homogenates were tested for the presence of the PEMT2 protein by immunoblotting with an antibody raised against the C-terminus of the rat PEMT2 protein. In both mouse and rat samples, bands were detected indicating a protein of approximately 22 kDa, close to the PEMT2 molecular weight of 22.5 kDa predicted above. These results closely match those predicted from the gene structure presented above.

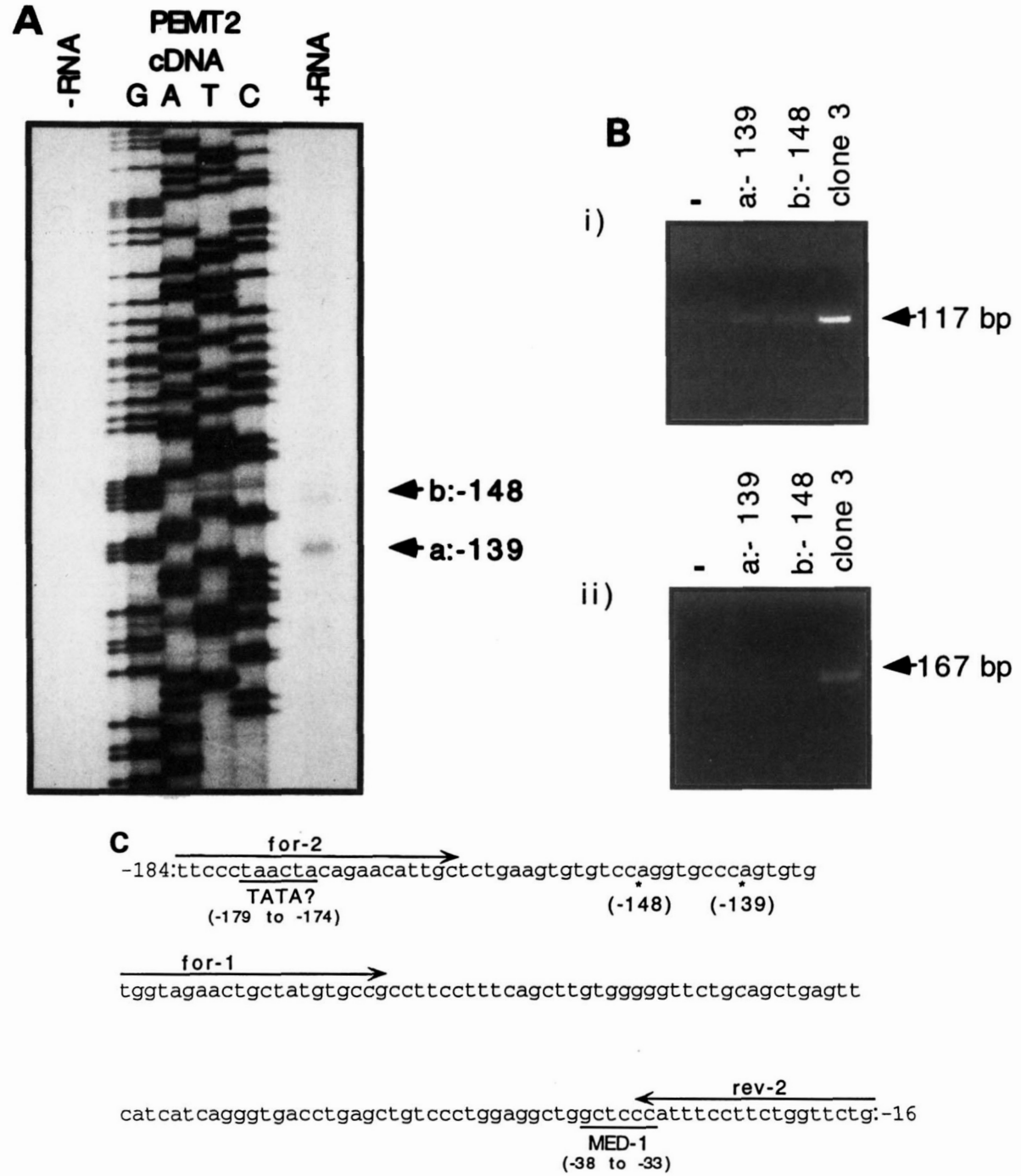

Fig. 4. Primer extension and reverse transcriptase-PCR results indicate two close transcription start sites. (A) RNA primer extension analyses were performed with and without mouse liver RNA (negative control) and rev-1, a 5′-labeled 30 mer oligonucleotide based on the complement of the putative mouse mRNA sequence. The sizes of the extension products were determined by comparison to a DNA sequencing ladder of rat PEMT2 cDNA using the same oligonucleotide primer. Nucleotide numbers of each transcription start site relative to the initiator methionine codon are indicated on the right. (B) PCR was performed on the extension products using the primers rev-2/for-1(i) and rev-2/for-2(ii). A region of the sequencing gel containing no extension products was used as a negative control. Clone 3 DNA was used as a positive control for the PCR reaction. (C) Sequence of the 5′ region of the PEMT2 gene. The transcription start sites are denoted by asterices. The TATA box region and the MED-1 sequence are underlined. The positions of the primers used in the PCR reactions are shown as arrows. The numbers refer to the positions of the various nucleotides relative to the initiator methionine codon.

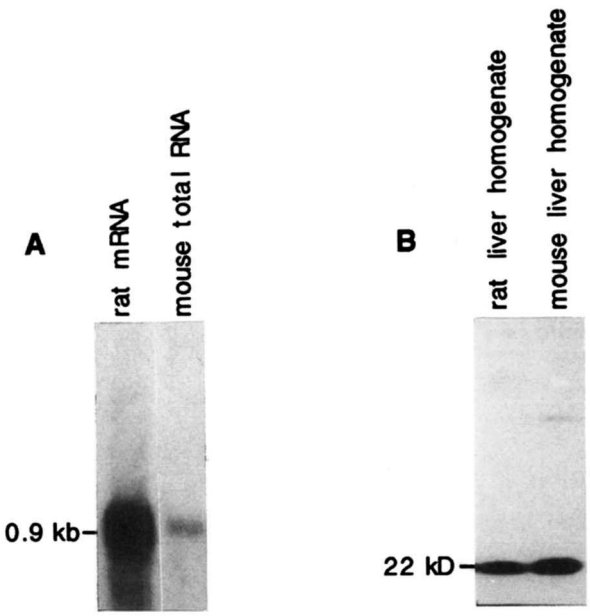

Fig. 5. (A) Northern blot of mouse liver total RNA and rat liver mRNA. Two-μg polyA-enriched RNA samples were isolated, electrophoresed, blotted, and probed with rat PEMT2 cDNA, as described in Materials and Methods. The size of the bands is indicated on the left. (B) SDS-polyacrylamide gel electrophoresis immunoblot of mouse and rat liver homogenates containing 50 μg total protein. Antibody raised against the C-terminus of rat PEMT2 detects 22 kD proteins from both rat and mouse homogenates.

Chromosomal localization of PEMT2

The mouse chromosomal location of the PEMT2 gene (designated as the *Pempt2* locus) was determined by the interspecific backcross mapping method (8). Female C57BL/6J strain mice were crossed to male *M. spretus* strain mice, producing heterozygous offspring. These two inbred strains of mice were chosen because each strain carries a different allele for thousands of genetic loci. One method for differentiating the alleles is through restriction fragment length polymorphisms, or RFLPs. When genomic DNA from the two strains is digested with a restriction enzyme and Southern blotted with a probe for a locus, a difference is seen in the hybridization pattern for the two strains. This difference reflects a change in the nucleotide sequence of the locus between the two strains. Despite this genetic divergence, female offspring of the cross are fertile.

The females of the F_1 generation were then backcrossed to males of the parental *M. spretus* strain, producing 94 N_2 offspring. Each offspring contained one set of *M. spretus* chromosomes from the father and one set of chromosomes from the heterozygous mother. The maternal chromosomes underwent recombination during meiosis, producing a "mosaic" of *M. spretus* and C57BL/6J segments. Therefore, at each locus, an N_2 offspring was either homozygous, with two *M. spretus* alleles, or heterozygous, with one *M. spretus* allele and one C57BL/6J allele. The genomic DNA of 94 N_2 offspring was isolated. For thousands of different loci whose positions were already known, the pattern of heterozygosity versus homozygosity of alleles (the segregation pattern) was determined in the N_2 generation. Each different locus produces its own pattern, reflecting the recombination points in the maternal chromosomes. Therefore, two loci closely linked will have a low frequency of recombination between them, producing nearly identical patterns of heterozygosity versus homozygosity.

To map the *Pempt2* locus, it was necessary to find a suitable RFLP. A selection of restriction enzymes was screened with genomic DNA from C57BL/6J and *M. spretus* mice in Southern blots probed with a 1.9 kb fragment of clone 11 containing exon 5. The restriction enzyme *Bgl2* produced a distinct RFLP: one 6.6 kb band in C57BL/6J DNA and two bands of 3.2 and 4.0 kb in *M. spretus* DNA (**Fig. 6a**). This RFLP was followed in the genomic DNA of the 94 N_2 progeny. The *Bgl2* RFLP pattern of the *Pempt2* locus matched exactly that of the marker *D11Bir9*. Significant linkage was also found with the marker *D11Mit4* and the *Csfgm* locus (for colony-stimulating factor, granulocyte-macrophage). These results place the *Pempt2* locus on mouse chromosome 11, approximately 31 centimorgans from the centromere (Fig. 6, b and c). The distances between *Pempt2* and nearby loci in centimorgans (±standard error), and the most likely gene order (proximal to distal), are: *Csfgm*–3.2(±1.8)–*Pempt2* and *D11Bir9*–4.3(±2.1)–*D11Mit4*. This region of mouse chromosome 11 is syntenic to human chromosomes 5q and 17p.

DISCUSSION

For phospholipid biosynthesis, murine PEMT2 is the first full-length mammalian gene that was cloned and characterized. Only portions of the genes for rat choline kinase and mouse CTP:phosphocholine cytidylyltransferase, have been isolated and characterized (12, 13). These results for the mouse PEMT2 gene will allow us to further investigate the role of phospholipid methylation in whole animals by gene disruption.

The PEMT2 gene contains seven exons in at least 35 kb, and exists as a single copy. The exons vary in size between 75 and 259 base pairs (Fig. 2). The introns range from 0.3 kb to greater than 13 kb (Table 1). The PEMT2 gene is more than 30-fold larger than the fully processed mRNA, although such a size difference is not particularly unusual (14). It will be interesting to learn whether other genes involved in phospholipid biosyn-

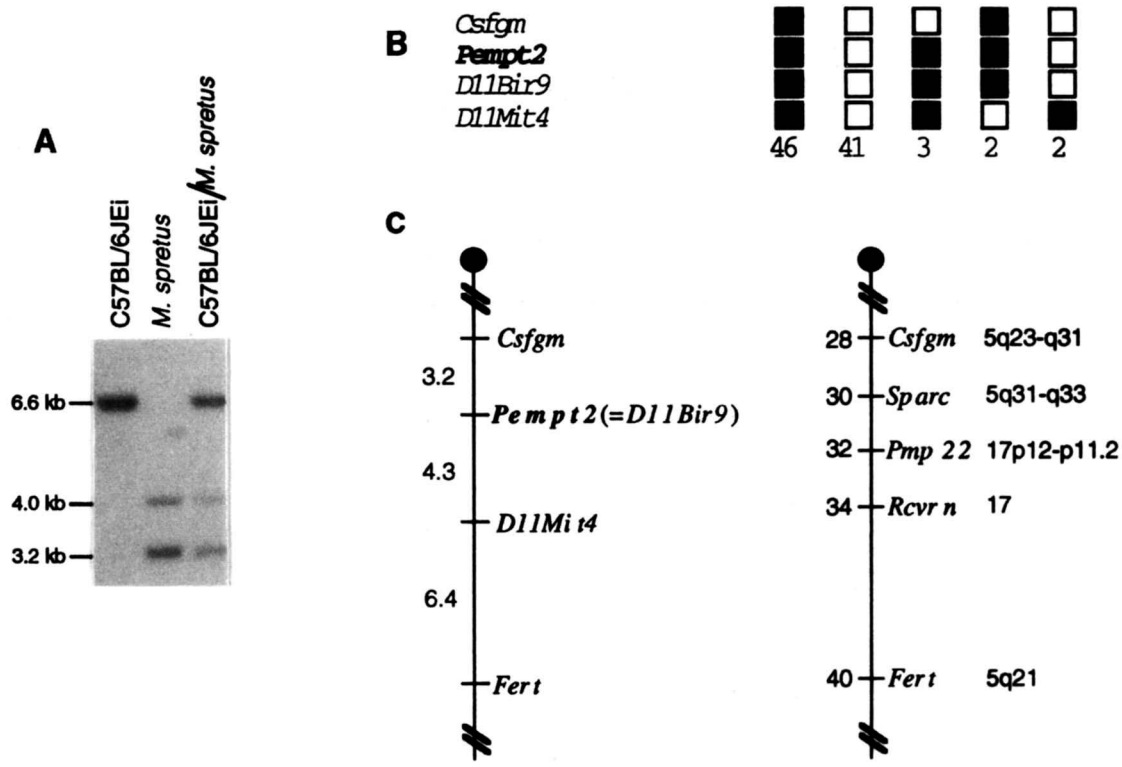

Fig. 6. *Pempt2* maps to mouse chromosome 11. (A) Southern blot of genomic DNA from C57BL/6JEi, *M. spretus* and an F_1 offspring of the two parental strains, using an exon 5-containing probe, reveals a polymorphism in the *Pempt2* locus. (B) The segregation patterns of the *Pempt2* polymorphism and flanking markers in 94 backcross progeny were determined. Each column represents chromosome 11 inherited from the (C57BL/6JEi × *M. spretus*) F_1 parent. Black boxes represent the C57BL/6JEi allele, and the white boxes represent the *M. spretus* allele. The number of offspring inheriting each type of chromosome is indicated at the bottom of each column. (C) The figure on the left is a partial map of mouse chromosome 11, showing the position of the *Pempt2* locus in relation to the nearby markers with which it was mapped. Recombination distances between the markers are shown in centimorgans on the left. The figure on the right is a partial composite linkage map of mouse chromosome 11 (data from GBASE, 1995, The Jackson Laboratory, Bar Harbor, ME). The numbers on the left indicate the distance of each locus from the centromere, in centimorgans. The positions of these same loci on human chromosomes are shown on the right.

thesis in mammals have a similar size. The mouse exons are >92% identical to the rat PEMT2 cDNA, suggesting strong conservation across species.

PEMT2 gene expression is regulated in both a tissue-dependent and developmental manner. The cloning of the PEMT2 gene, and in particular its 5′ end, forms a foundation for the analysis of *cis*-acting elements that regulate its expression. Sequence analysis does not reveal a consensus TATA box sequence approximately 30 bp upstream of the 5′-most transcription start site. The presence of a functional TATA box in such a position would suggest that PEMT2 is a transcriptionally regulated gene, rather than a constitutively expressed "housekeeping" gene (15). However, multiple transcription start sites and the presence of the MED-1 consensus sequence (GCTCCC) 110 bp downstream of the first start site place PEMT2 into a recently described family of TATA-less RNA polymerase II promoters (Fig. 4c) (16).

In the liver, the first appearance of PEMT2 mRNA occurs perinatally (Z. Cui and D. E. Vance, unpublished observation). Several other liver-specific genes are expressed in a similar pattern, including tyrosine aminotransferase (17) and α_1-antitrypsin (18). Liver-specific transcription factors, including members of the HNF (Hepatocyte Nuclear Factor) family and C/EBP (CCAAT/Enhancer binding protein), have been implicated in gene expression during development (19). **Figure 7** shows potential transcription factor binding sites identified in the immediate 5′ upstream region of the mouse PEMT2 gene. Interestingly, there are putative binding sites for both HNF5 and C/EBP. The role of these and other potential *cis*-acting elements in PEMT2 gene expression will be a focus of further investigation.

Using backcross mapping, the mouse *Pempt2* locus was localized to chromosome 11, approximately 31 centimorgans distal to the centromere. In contrast, the CTP:phosphocholine cytidylyltransferase gene was localized to mouse chromosome 16 (12). The same markers on mouse chromosome 11 that surround the *Pempt2*

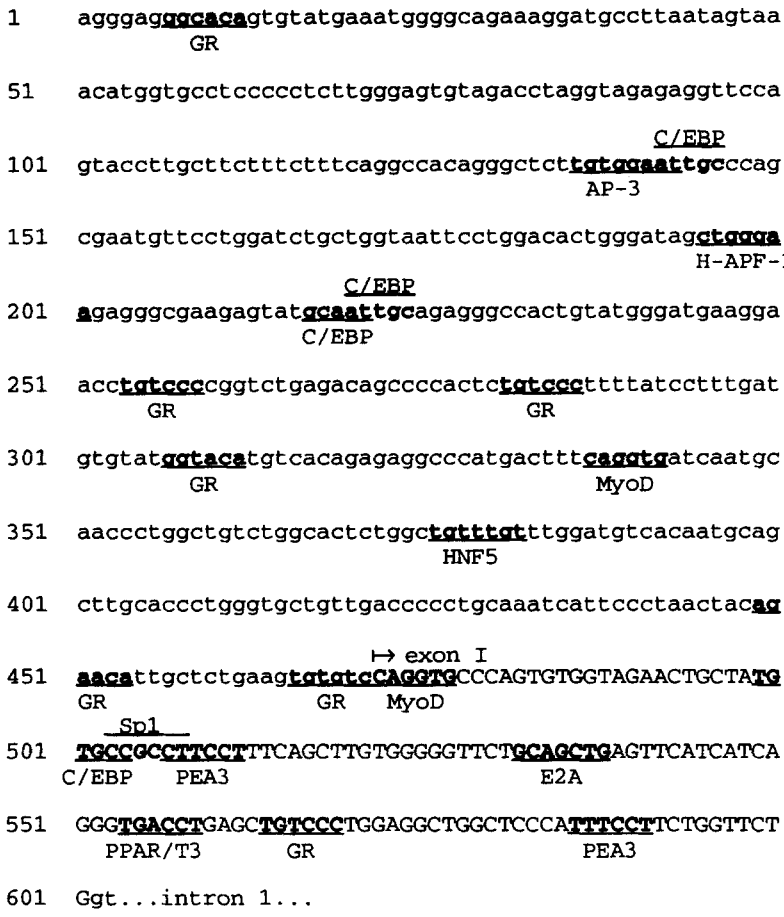

Fig. 7. Sequence analysis of the murine PEMT2 exon I (capitalized) and its 5′-flanking region. Candidate binding sites for transcription factors were identified using the *Findpatterns* program of the GCG sequence analysis software package (Genetics Computer Group, Inc., Madison, WI), with a combined database from TFD (release 7.5, 3/96) and Transfac (release 2.5, 1/96). AP-3: binding site for AP3; C/EBP: binding site for the CCAAT/Enhancer binding protein; E2A: E box binding site for E2A; GR: glucocorticoid receptor binding element; H-APF-1: interleukin-6 responding element; HNF5: binding site for Hepatic Nuclear Factor 5; MyoD: binding site for MyoD; PEA3: primary target of signal transduction responding to TPA, EGF and serum; PPAR: binding site for the Peroxisome Proliferator Activated Receptor; T3: binding site for the Thyroid Hormone Receptor.

locus have their human counterparts on either chromosome 5q or 17p. Both 5q and 17p have been characterized as mutational "hot spots", frequently deleted in primary liver cancer (20). Therefore, we shall localize the PEMT2 gene in human chromosomes to investigate the role of this enzyme in human hepatocarcinogenesis.

We compared our map of chromosome 11 to a composite map showing the chromosomal location of many uncloned mouse mutations, in the hopes of finding a pre-existing mouse line carrying a mutated PEMT2 gene. The region of chromosome 11 near the *Pempt2* locus does not contain any mouse mutations that suggest a defect in phospholipid metabolism or liver growth. Defective mitochondria-associated membrane and low levels of PEMT2 protein were detected in mice carrying the *mnd* (for Motor Neuron Degeneration) gene (J. E. Vance and J. R. Faust, unpublished observation). These mice are a model for neuronal ceroid lipofuscinosis, or Batten's disease, which is characterized by defective lipid storage in neurons (21, 22). However, the *mnd* gene maps to the proximal end of mouse chromosome 8 (23), not 11, strongly suggesting that a fault in the PEMT2 gene is not the primary cause of the *mnd* defect. Therefore, further investigation is required to determine the link between decreased PEMT2 protein expression and the *mnd* phenotype.

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REFERENCES

1. Bremer, J., and D. M. Greenberg. 1961. Methyl transfering enzyme system of microsomes in the biosynthesis of lecithin (phosphatidylcholine). *Biochim. Biophys. Acta.* **46:** 205–216.
2. Cui, Z., J. E. Vance, M. H. Chen, D. R. Voelker, and D. E. Vance. 1993. Cloning and expression of a novel phosphatidylethanolamine *N*-methyltransferase. A spe-

cific biochemical and cytological marker for a unique membrane fraction in rat liver. *J. Biol. Chem.* **268:** 16655–16663.
3. Pelech, S. L., E. Power, and D. E. Vance. 1983. Activities of the phosphatidylcholine biosynthetic enzymes in rat liver during development. *Can. J. Biochem. Cell Biol.* **61:** 1147–1152.
4. Grisham, J. W. 1962. A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H^3. *Cancer Res.* **22:** 842–849.
5. Cui, Z., M. Houweling, and D. E. Vance. 1994. Suppression of rat hepatoma cell growth by expression of phosphatidylethanolamine *N*-methyltransferase-2. *J. Biol. Chem.* **269:** 24531–24533.
6. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
7. Kingston, R. E. 1987. Preparation and analysis of RNA. *In* Current Protocols in Molecular Biology. John Wiley & Sons, New York, NY. 4.1.1–4.10.8.
8. Rowe, L. B., J. H. Nadeau, R. Turner, W. N. Frankel, V. A. Letts, J. T. Eppig, M. S. Ko, S. J. Thurston, and E. H. Birkenmeier. 1994. Maps from two interspecific backcross DNA panels available as a community genetic mapping resource. *Mamm. Genome.* **5:** 253–274.
9. Mount, S. M. 1982. A catalogue of splice junction sequences. *Nucleic Acids Res.* **10:** 459–472.
10. Simon, M. C., T. M. Fisch, B. J. Benecke, J. R. Nevins, and N. Heintz. 1988. Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for E1A regulation. *Cell.* **52:** 723–729.
11. Wefald, F. C., B. H. Devlin, and R. S. Williams. 1990. Functional heterogeneity of mammalian TATA box sequence revealed by interaction with a cell-specific enhancer. *Nature.* **344:** 260–262.
12. Rutherford, M. S., C. O. Rock, N. A. Jenkins, D. J. Gilbert, T. G. Tessner, N. G. Copeland, and S. Jackowski. 1993. The gene for murine CTP:phosphocholine cytidylyltransferase (Cptct) is located on mouse chromosome 16. *Genomics.* **18:** 698–701.
13. Uchida, T. 1994. Regulation of choline kinase R: analyses of alternatively spliced choline kinases and the promoter region. *J. Biochem. (Tokyo).* **116:** 508–518.
14. Watson, J. D., N. H. Hopkins, J. W. Roberts, J. A. Steitz, and A. M. Weiner. 1987. Molecular Biology of the Gene. Benjamin/Cummings, Menlo Park, CA. 628.
15. Kageyama, R., G. T. Merlino, and I. Pastan. 1989. Nuclear factor ETF specifically stimulates transcription from promoters without a TATA box. *J. Biol. Chem.* **264:** 15508–15514.
16. Ince, T. A., and K. W. Scotto. 1995. A conserved downstream element defines a new class of RNA polymerase II promoters. *J. Biol. Chem.* **270:** 30249–30252.
17. Nitsch, D., M. Boshart, and G. Schultz. 1993. Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements. *Proc. Natl. Acad. Sci. USA.* **90:** 5479–5483.
18. Courtois, G., J. G. Morgan, L. A. Campbell, G. Fourel, and G. R. Crabtree. 1987. Interaction of a liver-specific nuclear factor with the fibrinogen and α1-antitrypsin promoters. *Science.* **238:** 688–692.
19. Xanthopoulos, K. G., and J. Mirkovitch. 1993. Gene regulation in rodent hepatocytes during development, differentiation and disease. *Eur. J. Biochem.* **216:** 353–360.
20. Ozturk, M. 1995. Molecular Biology and Biotechnology. VCH, New York, NY. 516–518.
21. Bronson, R. T., B. D. Lake, S. Cook, S. Taylor, and M. T. Davisson. 1993. Motor neuron degeneration of mice is a model of neuronal ceroid lipofuscinosis (Batten's disease). *Ann. Neurol.* **33:** 381–385.
22. Chang, B., R. T. Bronson, N. L. Hawes, T. H. Roderick, C. Peng, G. S. Hageman, and J. R. Heckenlively. 1994. Retinal degeneration in motor neuron degeneration: a mouse model of ceroid lipofuscinosis. *Inv. Opthalmol. & Vis. Sci.* **35:** 1071–1076.
23. Messer, A., J. Plummer, P. Maskin, J. M. Coffin, and W. N. Frankel. 1992. Mapping of the motor neuron degeneration (Mnd) gene: a mouse model of amylotrophic lateral sclerosis (ALS). *Genomics.* **13:** 797–802.