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A B2 Repeat Insertion Generates Alternate Structures of the Mouse Muscle γ-Phosphorylase Kinase Gene

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A variety of cDNA and genomic clones for the γ-subunit of mouse muscle phosphorylase kinase (Phk-γM) have been isolated and characterized. The murine gene for Phk-γM (*Phkg*) exhibits multiple transcription start sites that are identical in skeletal muscle, cardiac muscle, and brain. The gene is composed of 10 exons and includes a 4.9-kb intron located in the 5′ untranslated region. Two mRNA species of 1.75 and 2.55 kb are produced from *Phkg* in ICR and C57BL/10 mice; these transcripts are colinear throughout the coding region and differ only in the length of the 3′ untranslated region. We have mapped the polyadenylation site of the 1.75-kb mRNA to the middle of exon 10; the 2.55-kb mRNA terminates further 3′ at the end of a mouse B2 repeat. In Balb/C mice an additional B2 insertion and related genomic rearrangements alter the sequence of *Phkg* exon 10 and are accompanied by an increase in the quantity of the 1.75-kb transcript and a decrease in the abundance and size of the longer transcript, from 2.55 to 2.35 kb. A PCR assay for sequences contained in exon 10 reveals that the Balb/C 3′ gene structure is shared by *Mus musculus castaneus* and *Mus musculus molossinus;* the C57BL/10 gene structure is shared by *Mus spretus*, *Mus domesticus*, and several strains of laboratory mice. These results suggest that *Phkg* in Balb/C mice was derived from *M. m. molossinus* and that *Phkg* of the other examined laboratory strains was derived from *M. domesticus*.

INTRODUCTION

Phosphorylase kinase (Phk; ATP:phosphorylase-b phosphotransferase, EC 2.7.1.38) is an intermediate enzyme in the glycogenolytic regulatory cascade and was the first regulatory protein kinase to be isolated (Krebs and Fischer, 1956). The Phk holoenzyme is composed of four molecules each of the four subunits α, β, γ, and δ. Phosphorylation of the α and β subunits via the β-adrenergic pathway and increased calcium concentrations, activate the enzyme (for review see Picket-Gies and Walsh, 1986). The calcium binding δ subunit is identical with calmodulin, an effector of several Ca^{2+}-dependent enzymes. The γ subunit catalyzes phosphorylation of the enzyme phosphorylase. Phk activity is highest in fast-twitch skeletal muscle but is also present in slow-twitch skeletal muscle (15% of the activity in fast-twitch muscle), cardiac muscle (5%), brain (5%), and liver (2%) (Gross and Mayer, 1974; Proux *et al.*, 1974; Cohen *et al.*, 1981).

There are several known isoforms for each of the Phk subunits, as well as several known genes that express calmodulin (Nojima, 1989). Three isoforms of the Phk α subunit have been cloned: αM and αM′, which are the alternatively spliced products of a single gene, *Phka1* (Zander *et al.*, 1988; Harmann *et al.*, 1991; Barnard *et al.*, 1990), and αL, which is the product of *Phka2* (Davidson *et al.*, 1992). The αL subunit is expressed primarily in brain and at lower levels in liver, cardiac muscle, and testis (Davidson *et al.*, 1992). The αM and αM′ subunits are predominant in fast-twitch glycolytic muscle fibers and in fast-twitch oxidative glycolytic and slow-twitch oxidative muscle fibers, respectively (Picket-Gies and Walsh, 1986; Pickett-Gies *et al.*, 1987). Two isoforms of the β-subunit have been identified, βM and βB; these are generated from a single gene by alternative splicing and are predominant in muscle and brain, respectively (Hartmann *et al.*, 1991; Kilimann *et al.*, 1988). The two known γ subunit isoforms, γM (Chamberlain *et al.*, 1987; Bender and Emerson, 1987) and γT (Hanks, 1989), are products of separate genes. Phk-γM is expressed at high levels in skeletal muscle and at lower levels in cardiac muscle and brain (Chamberlain *et al.*, 1987). Phk-γT is expressed predominantly in testis (Calalb *et al.*, 1992).

Although the αM and αM′ proteins were first recognized as alternate subunits in the early 1970s (Jennissen and Heilmeyer, 1974), the molecular mechanisms controlling the expression of these and other Phk subunits remain undetermined. The availability of gene sequence information for these isoforms will facilitate the search

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for shared regulatory motifs and common regulatory mechanisms. As a first step in understanding the diversity of phosphorylase kinase isoforms we report here the complete structure of the gene for Phk-γM in Balb/C mice. This research lays the groundwork for future studies on the mechanism of transcription control of the phosphorylase kinase subunit isoforms.

During the course of this work we observed an interesting example of gene evolution mediated by the insertion of mouse B2 repeat sequences. B2 repeats are present in $5–10 \times 10^5$ copies per genome and have been identified at the 3′ end of several mRNAs (Ryskov *et al.*, 1983; Kress *et al.*, 1984). *Phkg* generates two transcripts that differ in the length of the 3′ untranslated region; the longer transcript terminates at the 3′ end of a B2 repeat. Comparison of the 3′ end of *Phkg* in several laboratory strains and species of mice has revealed a second, proximal B2 insertion and related rearrangements in some strains of mice. These observations have allowed us to infer genetic relationships between these mice and propose a model for the evolution of *Phkg* by sequential insertion of B2 repeats.

MATERIALS AND METHODS

Isolation of Phk-γM cDNA clones. Full-length cDNA clones were isolated from an ICR mouse skeletal muscle cDNA library in λgt11 that was prepared by random hexamer and oligo(dT) priming of poly(A)$^+$ RNA (Chamberlain *et al.*, 1988b). Primary clones (2×10^6) were hybridized in duplicate with subclones of the 5′- and 3′-most ends of previously described mouse muscle Phk-γM cDNAs (Chamberlain *et al.*, 1987). Hybridization conditions were as described (Chamberlain *et al.*, 1987). Seven clones hybridized with both probes; these were plaque purified and the inserts were subcloned into pTZ19r (Pharmacia). Restriction analysis indicated that none of the clones contained sequences further 5′ than had been isolated previously, but that all contained the entire 3′ untranslated region for the longer of the two γ subunit mRNAs. The complete sequence of the 3′ untranslated region for one of these clones, γ-Phk5.1, was determined from subcloned fragments in the vectors pTZ19r or M13mp19 either manually or on an Applied Biosystems Model 373 automated DNA sequencer, as described previously (Chamberlain *et al.*, 1988a).

Isolation of Phkg genomic clones. Genomic clones for the γ subunit of mouse muscle Phk were isolated from a Balb/C mouse genomic library prepared by partial *Sau*3A digestion of genomic DNA followed by ligation into the vector λEMBL3. Plaques (10^6) were screened by hybridization with purified insert from the γM-Phk3 cDNA clone (Chamberlain *et al.*, 1987) as described previously (Chamberlain *et al.*, 1988a). To isolate the 3′ end of the gene, a second genomic library was prepared in λEMBL4 from *Eco*RI-digested Balb/C DNA size selected for restriction fragments between 12 and 16 kb in size. This latter library was screened with a 0.6-kb *Pvu*II fragment from the 3′ end of the Phk-γM cDNA, and eight identical clones containing a 14-kb *Eco*RI fragment were isolated (λPhkE14, Fig. 1A). The *Phkg* genomic clones were restriction mapped with eight enzymes (Fig. 1A). Exon-containing restriction fragments were subcloned into pUC18 and pUC19, pTZ18 and pTZ19, or M13mp18 and M13mp19 and sequenced on an Applied Biosystems Model 373 automated DNA sequencer (Figs. 1A and 2). Selected genomic fragments were amplified by polymerase chain reaction (PCR) and cycle sequenced using the Taquence v.2.0 kit (USB). For sequencing, PCR products were electrophoresed through agarose gels, isolated from gel slices by centrifugation through sterile glass wool, precipitated with ethanol, and resuspended in H_2O. Cycle sequencing reactions contained 100 ng each of purified PCR product and either the forward or reverse PCR primer and were carried out using PCR amplification conditions (see below) for 40 cycles. Genomic DNA sequences were aligned with cDNA sequences and with the GenBank database to identify exon/intron boundaries and internal repeats using the MacVector program (IBI). cDNA and genomic clone sequence data have been deposited in GenBank.

RNA analysis. Total RNA was isolated from mouse tissues as described previously (Chomczynski and Sacchi, 1987), reextracted with phenol/chloroform, and ethanol precipitated. Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography (Aviv and Leder, 1972). For Northern blot analysis, 10 μg of total RNA was resolved by electrophoresis through a 1.35% agarose/0.66 *M* formaldehyde gel, transferred to Nytran membrane (Schleicher and Schuell), and fixed using a UV crosslinker (Stratagene). Membranes were prehybridized at 42°C in 5× SSC (1× SSC = 150 m*M* NaCl, 15 m*M* citric acid, pH 7.0), 5× Denhardt's solution [1× Denhardt's = 0.2 mg/ml each Ficoll, polyvinylpyrrolidone, and bovine serum albumin], 0.05 *M* Tris (pH 7.5), 0.1% sodium pyrophosphate, 0.5% sodium dodecyl sulfate (SDS), 50% formamide, and 100 μg/ml each herring sperm DNA, poly (dATP) and poly (dCTP). Hybridization conditions were as above, except that the concentrations of Denhardt's solution and SDS were 1× and 0.1% respectively, and the poly(dATP) and poly(dCTP) were omitted. cDNA probes were labeled using a Prime-It II kit (Stratagene) and added to the hybridizations at $1–2 \times 10^6$ cpm/ml. Filters were washed for 1 h at 60°C in 2× SSC and 0.5% SDS followed by 20 min at 45°C in 0.5× SSC and 0.1% SDS (for Phk-γM) or 0.1× SSC and 0.1% SDS (for MCK). Filters were regenerated after hybridization with Phk-γM by washing for 60 min at 65°C in 50% formamide, 0.1% SDS, and 4× SSPE [1× SSPE = 150 m*M* NaCl, 10 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.4]. The amount of hybridization obtained with the MCK probe was measured with a Betascope imager (Betagen). Densitometry of the Northern analysis autoradiograms was performed with a BioImage gel analysis system (Millipore).

Reverse transcription and PCR. Single-stranded cDNA for PCR was prepared by reverse transcription (RT) of total RNA. A 15.5-μl reaction containing 1 μg total RNA, 10 μg random hexamers (Pharmacia), 2 μg oligo$(dT)_{17}$, and 4 μl 5× RT buffer [1× RT buffer = 50 m*M* Tris–HCl (pH 8.3 at 37°C), 8 m*M* $MgCl_2$, 30 m*M* KCl, and 10 m*M* dithiothreitol (DTT)] was heated to 95°C for 1 min and then chilled on ice. The reaction volume was increased to 20 μl with the addition of 50 nmol each dNTP, 2 μg BSA, 10 units of RNAsin (Pharmacia), and 200 units of SuperScript MMLV reverse transcriptase (BRL). The reactions were incubated at 37°C for 1 h and stored at −70°C. Twenty-five-microliter PCRs contained 0.2 μ*M* primers, 1 U *Taq* polymerase (Engelke *et al.*, 1990), and 0.2 m*M* each dNTP, in 1× reaction buffer [10 m*M* Tris–HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$]. PCR templates were 1 ng cloned DNA, 125 ng genomic DNA, or 1 μl reverse transcribed first-strand cDNA. Amplification was performed on a Perkin–Elmer Cetus thermocycler using the step cycle function. The reactions were heated to 94°C for 4 min, amplified for 30 to 35 cycles, and incubated for 5 min at 72°C. Amplification cycles were 30 s denaturation at 94°C, 30 s annealing (optimum temperature varies), and 30 s minimum extension at 72°C. The annealing temperature was optimized for each primer set and the extension time was increased by 30 s for each 500 bp to be amplified beyond 500 bp.

Rapid amplification of cDNA ends (RACE). The following modifications were made to the published procedure (Frohman 1990). Reverse transcription was performed as described above except that 50 ng poly(A)$^+$ RNA was used as template and 500 ng of an oligo(dT)-adapter primer, Ad(dT) [5′ GGTCAGCTGATGTGCAAGG$(T)_{15}$ 3′], replaced both the random hexamers and the oligo$(dT)_{17}$. One hundred-microliter PCRs were assembled in two layers using AmpliWax PCR Gems (Perkin–Elmer). The 25-μl lower layer contained 0.8× PCR buffer, 4 U *Taq* polymerase, 200 ng Phk-γM forward primer F4 (Fig. 2B), and 200 ng of the adapter primer without oligo(dT) sequence. The 75-μl top layer contained 1.1× PCR buffer, 8 μl of 2.5 m*M* dNTPs, and 2 μl of the RACE first-strand cDNA. PCR was performed as described above using an annealing temperature of 58°C and 1-min extensions for 35 cycles. The gel-purified product was treated with T4 DNA polymerase and ligated into the *Hin*cII site of pTZ19. Following transformation of *Escherichia coli*, plasmid inserts from individual colonies were amplified by PCR using vector-specific primers and cycle sequenced.

Primer extension. Primer extensions were performed essentially as described (Boorstein and Craig, 1989). Synthetic oligonucleotides PXA (5′ ATCCCAAACACCTCGTGAGGTCCTTGTAGG 3′) and PXB (5′ AGTGACAAGAATTCCAAAGGTCCTGGAGCC 3′), complementary to the 5′ untranslated region of Phk-γM, were end-labeled with T4 polynucleotide kinase. Primer (5×10^4 cpm) was annealed with 2 μg poly(A)$^+$ RNA, 3 μg tRNA, or 25 pg synthetic-sense RNA (see below) in 10 μl of 1× single-strand hybridization buffer [0.3 *M* NaCl, 10 m*M* Tris–HCl, pH 7.5, and 2 m*M* EDTA]. After incubation for 3 h at 50°C, 40 μl of 1.25× transcription buffer [1× transcription buffer = 1 m*M* dNTPs, 50 m*M* Tris (pH 8.3 at 37°C), 8 m*M* $MgCl_2$, 30 m*M* KCl, 10 m*M* DTT, 100 μg/ml BSA, 100 μg/ml Actinomycin C1] and 300 U of MMLV reverse transcriptase were added to each reaction and the mixture was incubated at 42°C for 1 h. The reactions were stopped by addition of 1 μl 0.5 *M* EDTA, ethanol precipitated, and resuspended in 2 μl H_2O and 5 μl formamide loading dye. Extension products were size fractionated on a denaturing 6% polyacrylamide gel and analyzed by autoradiography.

RNase protection. Exon 1 and upstream sequences were PCR amplified from Balb/C genomic DNA using primers F0 and R1 (Fig. 2A). Reactions were treated with T4 DNA polymerase, gel purified using the MerMaid kit (Bio 101), ligated into the *Hinc*II site of pTZ19R, and sequenced as described above. Plasmids pPhkN and pPhkR contained the insert in opposite orientations and were used to generate sense and antisense RNA, respectively. RNase protection analysis was performed using antisense RNA from plasmid pPhkR as described previously (Chamberlain *et al.*, 1988b). Sense RNA from plasmid pPhkN was used for the primer extension reactions.

RESULTS

Isolation of Genomic Clones for Phk-γM

Genomic clones for mouse muscle Phk-γM were isolated by screening a Balb/C genomic library. Five clones were identified by hybridization with the cDNA subclone γM-Phk3 (Chamberlain *et al.*, 1987), of which two were found to be identical. These clones were restriction mapped with a variety of enzymes, and exon-containing restriction fragments were subcloned and sequenced (Figs. 1 and 2). This analysis revealed that none of the genomic clones contained the 3′ end of the gene, and rescreening of the library indicated that the 3′ end of the gene was not present in the original library. Southern analysis of Balb/C genomic DNA revealed that the 3′ end of the gene was present on a 14-kb *Eco*RI fragment (data not shown). Therefore, we prepared a size-selected *Eco*RI total digest genomic library from which the 3′ end of the gene was isolated. Figure 1A is a schematic illustration of the gene for Phk-γM from Balb/C mice. The location of all restriction sites for eight enzymes, the location of the exons and sequenced regions, the extent of each of the genomic clones analyzed, and the location of various B1 and B2 repetitive elements (some of which are truncated) within the sequenced regions are indicated on the figure. Figure 2 displays the sequence of the exons and flanking regions.

Exon boundaries of the gene were determined initially by comparison of sequence data of cDNA clones isolated from C57BL/10 and ICR skeletal muscle cDNA libraries (Chamberlain *et al.*, 1987; Bender and Emerson, 1987). These data indicated that the gene was divided into 10 exons and 9 introns (Figs. 1 and 2) flanked by consensus splice donor or acceptor sequences [Table 1; (Mount, 1982)]. However, this analysis was complicated by the presence of two insertions of 165 and 209 bp within the 3′ untranslated region in exon 10 of the Balb/C gene relative to the sequence of cDNA clones isolated from two independent ICR mouse libraries [Figs. 1B and 2; Bender and Emerson, 1987; this work]. This analysis also revealed a short tandem repeat (STR) polymorphism of $(T)_{19}(A)_3$ vs $(T)_{10}(A)_2$ in Balb/C and ICR mice, respectively. The inserted sequences did not appear to represent introns as they were not flanked by consensus splice donor and acceptor sequences (Fig. 2); this suggested that the Balb/C and ICR mouse genes for Phk-γM might differ in their 3′ end structure.

3′ End Structure of Phkg

The 3′ end structure of *Phkg* was compared in a variety of different mouse species and strains by PCR amplification from genomic DNA and reverse transcribed mRNA. PCR was performed with primers F5 and R5 (Fig. 2B), which are complementary to sequences separated by 200 bp in the ICR cDNA and by 585 bp in Balb/C genomic DNA. A 585-bp PCR product was obtained with Balb/C and *Mus molossinus* genomic DNAs, whereas the product from *Mus castaneus* (CASA/RK) appeared slightly smaller (Fig. 3). In contrast, a PCR product of 200 bp was obtained with genomic DNA from six laboratory strains of mice and from both *Mus spretus* and *Mus domesticus*. The 200-bp product comigrated with the PCR product obtained from reverse transcribed C57BL/10 RNA and ICR mouse cDNA clones (Fig. 3 and data not shown). We conclude that *Phkg* displays two types of 3′ end structure: a shorter version as exemplified by the C57BL/10 and ICR genes (class I *Phkg*) and a longer version that contains insertions totaling 385 bp and which is found in the Balb/C gene (class II *Phkg*). Cycle sequencing of the PCR products from several mouse strains with the class I or the class II gene structure revealed that the sequences between primers F5 and R5 were nearly identical for each class. Only a few base changes were observed between any two mouse strains with a given gene class. The CASA/RK sequence, however, was 20 bp shorter than those of Balb/C and *M. m. molossinus* due to a tetranucleotide repeat length variation of $(TAAA)_2$ vs $(TAAA)_7$. This length variation is at the 3′ end of a mouse B2 repeat in exon 10 and is similar to other polymorphic STRs present in a variety of eukaryotic species [(Beckman and Weber, 1992); Figs 1B and 2B].

To explore the nature of the sequences inserted into the class II gene, the ICR cDNA and Balb/C genomic sequences were compared with sequences in the GenBank database and also aligned to detect self-complementarity. This analysis identified a number of B1 and B2 repeats within the gene (Figs. 1 and 2) as well as a B2 repeat at the very 3′ end of the ICR mouse cDNA (Fig. 1B). Colinearity between the class I and the class II genes is interrupted by two insertions into exon 10 of the class II gene, and these insertions occur at the exact same positions within each class II gene sequenced (Figs. 1B and 2). Outside of these insertions the ICR and Balb/

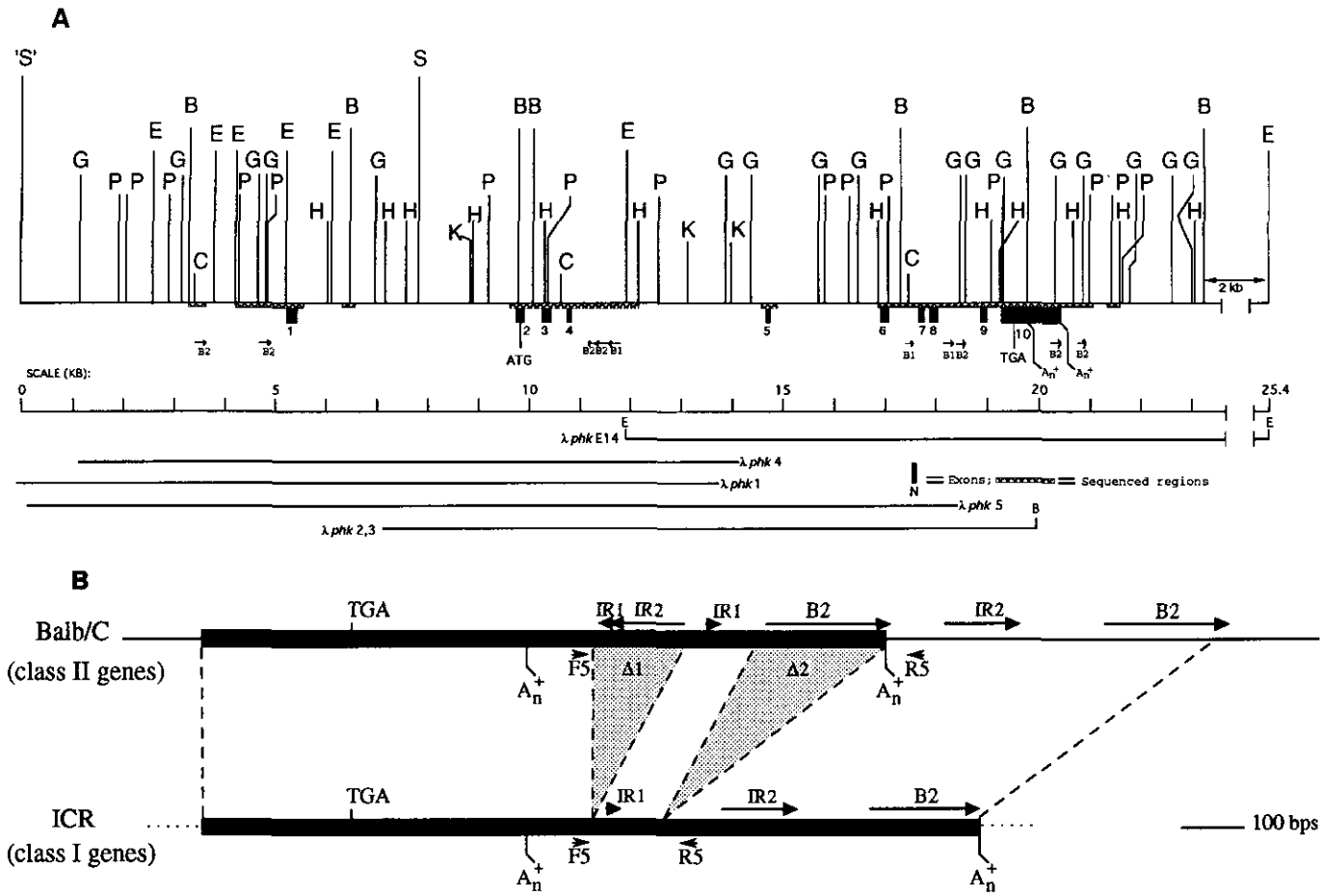

FIG. 1. (**A**) Structure of the Balb/C *Phk-γM* gene. Sequenced regions are indicated by shaded bars, exons by solid boxes. Location and 5′ to 3′ orientation of B1 and B2 repeats are indicated with arrows. Lambda clones are aligned beneath the restriction map. Restriction enzyme sites are abbreviated as follows: S, *Sal*I; B, *Bam*HI; E, *Eco*RI; G, *Bgl*II, P, *Pst*I; H, *Hin*dIII; K, *Kpn*I; C, *Sac*I. (**B**) Comparison of the class I (ICR and C57BL/10) and class II (Balb/C) *Phk-γM* gene structures. Exon 10 is represented by heavy lines; flanking sequences are indicated by thin lines; unsequenced flanking regions are indicated by dots. Dashes indicate boundaries of known sequence identity in the Balb/C and ICR genes. The location and orientations of inverted repeats and B2 sequences are shown by arrows above the line, PCR primers are indicated below the line. Also shown are the location of the polyadenylation addition sites and the stop codon. Δ1 and Δ2 indicate sequences present in the Balb/C gene but not in the ICR gene (Fig. 2B).

C sequences maintain a high degree of sequence identity: single base changes within the coding region are described in the legend to Fig. 2, whereas similarities in the 3′ untranslated region are as follows. In the region between the stop codon and the first insertion (Δ1) of the Balb/C gene 404/413 bases are identical (98%) between the Balb/C and ICR sequences; this is followed by 6 bases of ICR sequence that have a 3/6 base match with either end of the Δ1 insertion. In the region between insertions Δ1 and Δ2 (114 bp in the Balb/c gene) the two classes of genes differ at one position and display an STR of $(T)_{19}(A)_3$ in Balb/C mice vs $(T)_{10}(A)_2$ in ICR mice. From the second insertion (Δ2) to the 3′ end of the ICR cDNA, 486/508 bases are identical (96%) between the ICR and Balb/c sequences.

Several features of the inserted sequences in the Balb/C exon 10 were also noted. The 5′-most insertion, Δ1, contains two adjacent stretches of sequence that represent inverted repeats of portions of the gene located further 3′. The 5′-most inverted repeat (designated IR1 in Fig. 2) begins 6 bp 3′ of the insertion and extends for 18 bp. The second inverted repeat (designated IR2 in Fig. 2) overlaps IR1 by 2 bp and extends for 119 bp. The 5′ copy of IR2 has two base mismatches and two single-base deletions relative to the complementary copy located 3′ of the insertion (Fig. 2) and is followed by 24 bases of unknown origin. The second insertion in the Balb/C gene is a 198-bp B2 repeat (Figs. 1B and 2) flanked by an 11-bp direct repeat.

The repetitive nature of the Balb/C insertions, as well as the absence of consensus splice junctions, suggested that these sequences might not be spliced from the Phk-γM mRNA transcript of the class II genes. To test this hypothesis, we synthesized two additional PCR primers, R3 and R4, which are complementary to sequences between F5 and R5 (Figs. 2B and 4). Primer R4 contains at its 5′ end three nucleotides that differ from the consensus mouse B2 sequence and was designed to anneal with the specific B2 repeat that is present in *Phkg* of Balb/C mice (Fig. 2B). Products of the predicted size are generated from Balb/C genomic DNA by amplification with primer F5 and each of the reverse primers R3, R4, and R5 (Figs. 2 and 4); however, when these same primer pairs were used to amplify reverse transcribed RNA, the

reaction with primer R5 failed to generate a product (Fig. 4). These results demonstrate that the sequences inserted into the Balb/C gene alter the 3′ end of the Phk-γM mRNA. The Balb/C messenger RNA is transcribed through the inserted sequences without splicing and terminates in the sequence between primers R4 and R5, most likely at the polyadenylation signal within the inserted B2 repeat. Figure 1B illustrates the deduced 3′ end structures of the class I and class II genes for Phk-γM.

The structure of the 3′ end of the class II gene suggests that the mature mRNA should be approximately 200 bp shorter in mice with the class II gene than in mice with the class I gene. To verify this prediction, total RNA from Balb/C and C57BL/10 mice was examined by northern analysis. Previous studies have demonstrated that two transcripts of 1.75 and 2.55 kb are generated from *Phkg* in ICR and C57BL/10 mice (Chamberlain *et al.*, 1987; Bender and Emerson, 1987). Figure 5 reveals that the larger of these transcripts is reduced in size by approximately 200 bp in Balb/C mice. The observed size difference between the longer Phk-γM transcripts is consistent with the altered exon 10 sequence in Balb/C mice.

Northern analysis also revealed that the 1.75-kb mRNA is expressed at higher levels in Balb/C than in C57BL/10 mice, and that the levels of the longer mRNA are reduced. The relative ratios of the Phk-γM mRNAs in Balb/C and C57BL/10 skeletal muscle were determined by densitometric analysis of autoradiograms obtained from three independent Northern blots, including the one displayed in Fig. 5. Rehybridization of the same blots with a mouse muscle creatine kinase (pMCKm36) cDNA (Jaynes *et al.*, 1986) enabled the relative amounts of the Phk-γM transcripts in the mouse strains to be normalized to the levels of MCK mRNA. These experiments revealed that the total amount of Phk-γM mRNA in Balb/C mice is 65% of that in C57BL/10 mice. In C57BL/10 mice the 1.75-kb transcript is 10% of the total Phk-γM mRNA, whereas in Balb/C mice it is 60% of the total. In addition, the 1.75-kb Phk-γM transcripts from both strains of mice were indistinguishable in size, suggesting that the same polyadenylation site is used for the shorter mRNA in both class I and class II genes.

Mapping the End of the 1.75-kb Phk-γM mRNA

Several groups have isolated cDNA clones for the larger of the two Phk-γM mRNAs (Chamberlain *et al.*, 1987; Bender and Emerson, 1987; da Cruz e Silva and Cohen, 1987; this study), but the site of polyadenylation for the shorter, less abundant, transcript has not been reported. It is difficult to predict precisely where the shorter transcript ends, as there are two separate polyadenylation signals (AATAAA) in exon 10 corresponding to the approximate size of the 1.75-kb mRNA (Fig. 2B). The 3′ end of the 1.75-kb transcript could not be mapped easily by RNase A protection, as the longer transcript would be expected to produce a high background of full-length probe protection. Therefore, the 3′ end of the shorter transcript was cloned from C57BL/10 mouse muscle using the RACE technique (see Materials and Methods). For this purpose an oligo(dT)-adapter primer was used to prime reverse transcription of muscle poly(A)$^+$ RNA. The single-stranded cDNA was PCR amplified with the Phk-γM-specific forward primer, F4 (Fig. 2B), and the adapter primer lacking the oligo(dT) sequence. A short extension time was utilized in the PCR to selectively amplify products from the short transcript. A single prominent band of PCR products was isolated from an agarose gel and subcloned.

Each of the sequenced RACE clones contained primer F4 and downstream Phk-γM sequences and was terminated with a stretch of adenine residues followed by the adapter primer. These results mapped the polyadenylation addition site to a location 15 bp downstream of the 5′-most AATAAA sequence in exon 10, and 5 to 12 bp upstream from two overlapping sequences similar to the consensus mRNA cleavage signal YGTGTTYY [(McLauchlan *et al.*, 1985); Fig. 2B and data not shown]. A few clones terminated within the overlapping downstream AATAAA sequences, and several additional clones ended with the sequence of the oligo(dT)-adapter primer in the upstream AATAAA sequence. These clones appear to have arisen by artifactual priming during reverse transcription, as in each case the adapter primer sequence was within a highly A-rich stretch of sequence and was not within 10–30 base 3′ of an AATAAA polyadenylation signal (Figs. 2B and data not shown).

Identification of the Phkg Transcription Start Site

The *Phkg* transcription start site was mapped by primer extension. Two primers were used: PXA, which overlaps the exon 1–2 boundary and is complementary to 17 bases in exon 1 and 13 bases in exon 2, and PXB, which is complementary to exon 1 upstream of primer PXA (Fig. 2A). Primer extension products were electrophoresed in parallel with sequencing reactions from a *Phkg* genomic subclone using PXA and PXB as sequencing primers, this allowed the RNA strand sequence to be read directly from the gel. Both primer extensions identified identical, multiple start sites in skeletal muscle (Fig. 6). The 5′-most start site was identified 1 bp downstream from a nearly perfect 18-bp palindromic sequence (Fig. 2A). A parallel reaction using synthetic RNA as template generated a single band of full-length extension and demonstrated that secondary structure in the RNA did not cause artifactual polymerase stopping. No extension products were detected with liver RNA, suggesting that *Phkg* is transcribed at a very low level, if at all, in liver.

Transcription start sites were also determined by RNase A protection of radiolabeled cRNA probes transcribed from the genomic subclone pPhkR (see Materials and Methods). These experiments also identified multiple transcription start sites, although the exact position of transcriptional initiation often varied by several bases from those determined by primer extension

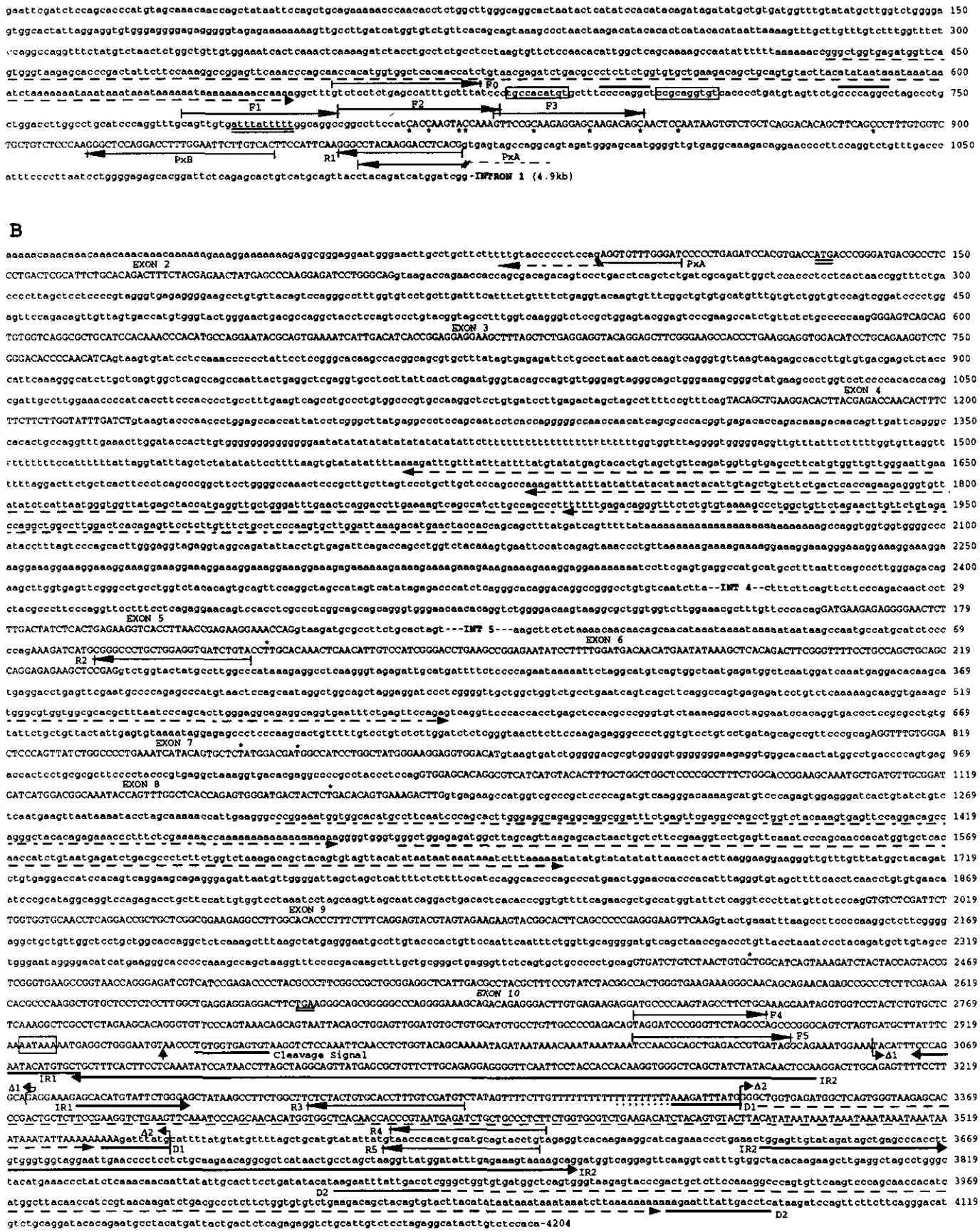

FIG. 2. Sequence of the Balb/C *Phk-γM* gene. Exons are written in uppercase letters and are indicated above the line of sequence. Introns and flanking sequences are written in lowercase. B2 repeats are underlined with dashes, and B1 repeats with alternating dots and dashes. Thin arrows and names designate the oligonucleotides used in PCRs and primer extensions; forward and reverse primers are indicated above and

(data not shown). This result likely reflects the cleavage specificity of RNase A, which has been noted previously (Sambrook *et al.*, 1989). Identical bands were obtained with RNase A protection of Balb/C and C57BL/10 skeletal muscle, demonstrating that transcription is initiated from identical sites in the two strains of mice (data not shown).

We also used an RT–PCR assay to confirm the transcription start sites in Balb/C skeletal muscle (Fig. 7). Three primers were chosen to bracket the transcription start sites: primer F1 is complementary to genomic sequences upstream of the mapped start sites, primer F2 overlaps the upstream start sites, and primer F3 overlaps the downstream sites (Figs. 2A and 7a). PCR conditions were optimized on genomic DNA using forward primers F1, F2, and F3 and reverse primer R1, which anneals with the 3′ end of exon 1. Each reaction produced a single band of the expected size (Fig. 7a). Primers F1, F2, and F3 were also used with reverse primer R2, which is complementary to sequences in exon 6 (Fig. 2B), to amplify reverse-transcribed RNA (Fig. 7b). Single fragments of the predicted size were generated with primers F2 and F3, revealing that Phk-γM transcripts are available to anneal with these primers; the absence of a product in the F1 to R2 reaction confirmed that primer F1 is upstream of the transcription start sites. Lowering the annealing temperatures in these PCRs did not increase the amount of product amplified by the upstream primers F1 or F2. These results confirmed the primer extension results and, together with the RNAse protections, demonstrated that identical transcription start sites are utilized in Balb/C and C57BL/10 skeletal muscle. Both the RNase protection and PCR assays were repeated on first-strand cDNA from C57BL/10 heart and brain, demonstrating that the same transcription start sites are used in heart, brain, and skeletal muscle (data not shown).

DISCUSSION

We have characterized the gene for Phk-γM from Balb/C mice and have compared the structure of the 3′ end of the gene between a variety of different types of mice. The Balb/C gene spans 16 kb and is composed of 10 exons. The 5′ untranslated region is encoded by exons 1 and 2 and the initiator codon is in exon 2. Exon 10 encodes the C-terminal 82 amino acids and a 3′ untranslated region of variable length. The first polyadenylation signal is 256 bases downstream of the stop codon; use of this signal results in a 1.75-kb mRNA that is the minor product of the class I genes and the major product of the class II genes. Polyadenylation signals at the 3′ ends of mouse B2 repeats produce mRNAs of 2.55 and 2.35 kb in class I and II *Phkg*, respectively.

Transcription from *Phkg* is initiated at multiple start sites that are identical in C57BL/10 and Balb/C skeletal muscle, cardiac muscle, and brain. Many other genes, including that of the recently described testis-specific isoform, Phk-γT, also display multiple start sites (Hanks, 1989; Melton *et al.*, 1984; Takenaka *et al.*, 1989; Martini *et al.*, 1986). Analysis of the Balb/C genomic sequences identified a nonconsensus TATA box approximately 21 bp upstream of the first transcription start site. It has been shown that mutations in the canonical TATAAA sequence can result in 5′ heterogeneity of transcripts without a significant decrease in the total level of transcription (Dynan and Tjian, 1985). An 18-bp imperfect palindrome, located between the TATA box and the first transcription start site, is suggestive of a protein dimer binding site. Within the region that we have sequenced there are several matches with the consensus binding site for the myogenic regulator MyoD [CANNTG (Lassar *et al.*, 1989)]. Two of these sequences (boxes, Fig. 2A) display 9 of 10 bp matches with the two adjacent MyoD binding sites in the muscle creatine kinase gene enhancer, and these sequences are separated by a similar distance in both genes (Buskin and Hauschka, 1989; Lassar *et al.*, 1989). Extensive functional testing of the promoter region will be necessary to prove whether these sequences in *Phkg* bind MyoD. No other muscle-specific regulatory sequences were identified; however, two perfect matches with the consensus Ap2 binding site are located 45 and 80 bp upstream of the TATA box (Fig. 2A).

Analysis of the alternative structures at the 3′ end of *Phkg* yielded several insights into the evolutionary history of this gene. Sequence data obtained from several strains of mice with either the class I or the class II gene structure indicated that both classes of genes are colinear and nearly identical outside of the insertions. Fur-

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below the line, respectively. These sequences have been deposited in GenBank and assigned the following Accession Nos. L08056, exon 1 and flanks; L08057, exons 2 through 4 and flanks; L08058, exon 5 and flanks; L08059, exons 6 through 10 and flanks. (**A**) Exon 1 and flanking sequence. Asterisks indicate the major transcription start sites. The nonconsensus TATA box is double underlined, and consensus Ap2 binding sites are overlined. Two 9/10 base matches with the MCK gene MyoD binding sites are boxed (Lassar *et al.*, 1989). A previously described ICR cDNA sequence (Bender and Emerson, 1987) includes an extra A 5 bases before the 3′ end of exon 1. (**B**) Exons 2 through 10 and flanking sequences. Sequence numbers begin again with number 1 after each discontinuity. Unsequenced introns are indicated within the line of sequence (INT). The initiator and stop codons are double underlined. A vertical arrow indicates the polyadenylation site mapped from the short transcript, which is preceded by a boxed polyadenylation addition signal and followed by an underlined consensus polyadenylation cleavage signal. Sequences present in the Balb/C gene but not in the ICR and C57BL/10 genes are labeled Δ1 and Δ2 and bracketed with arrows (see also Fig. 1B). Inverted repeats are denoted by thick arrows and are labeled IR1 and IR2. Direct repeats flanking two B2 repeats are also indicated by thick arrows and are designated D1 and D2. Single-base polymorphisms identified among the C57BL/10, ICR, and Balb/C amino acid coding sequences are indicated with asterisks: Exon 6, T in Balb/C, C in C57BL/10 and ICR; Exon 7 (both differences), T in Balb/C, C in C57BL/10 and ICR, the 5′-most difference encodes a Phe in Balb/C and a Ser in C57BL/10 and ICR; Exon 8, T in Balb/C and C57BL/10, C in ICR (only the change indicated alters the amino acid encoded). In addition, we have identified a sequencing error in our previously reported Phk-γM cDNA clone from C57BL/10 mice (Chamberlain *et al.*, 1987); a C in exon 10 was incorrectly reported as a G (asterisk).

TABLE 1

Exon–Intron Boundaries of the Murine *Phk-γM* Gene[a]

Intron	5′ Boundary	Size	3′ Boundary
1	CTCACG gtgagt −35 —	4.7 kb	ccccctccag AGGTGT −34 —
2	G GGC AG gtaaga 83 Arg (28)	376 bp	ctgccccaag G GGA GT 84 Arg (28)
3	AC ATC A gtaagt 262 Ile (88)	398 bp	tccgtttcag TA CAG C 263 Ile (88)
4	T GAT CT gtaagt 317 Leu (106)	3.8 kb	gttcccacag G ATG AA 318 Leu (106)
5	A ACC AG gtaaga 383 Arg (128)	2.2 kb	ctccctccag A AAG AT 384 Arg (128)
6	TC CGA G gtctgg 547 Glu (183)	570 bp	gttcccgcag AG GTT T 548 Glu (183)
7	G GAC AT gtaagt 638 Met (213)	137 bp	taccctccag G TGG AG 639 Met (213)
8	GAC TTG gtgaga 792 Leu (264)	817 bp	gttctcccag GTG TCT 793 Val (265)
9	TTC AAG gtactg 918 Lys (306)	286 bp	ccccctgcag GTG ATC 919 Val (307)

[a] Exon sequences are shown in uppercase, intron sequences in lowercase. Immediately below the exon sequence is indicated the base number of the first or last base of each exon. This numbering begins with the A of the initiator methionine as base 1. The amino acid encoded by the first and last codon of each exon is listed below the base number, and the position of the amino acid is indicated in parentheses.

thermore, all mice with the class II gene contain two insertions of virtually identical sequence that are located at precisely the same positions of exon 10, strongly

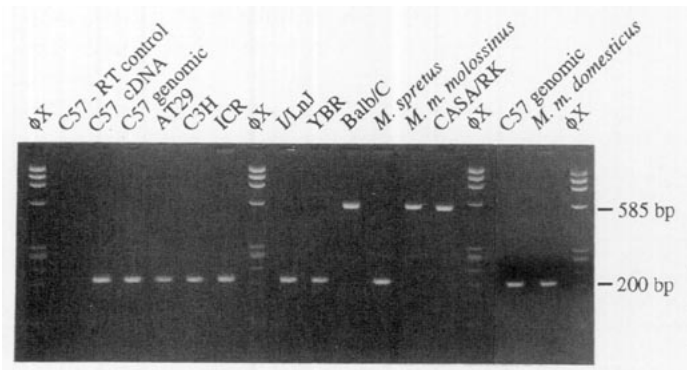

FIG. 3. Distribution of the class I and class II *Phkg* structures. Reverse-transcribed C57BL/10 RNA and various genomic DNAs were PCR amplified using primers F5 and R5 (see Fig. 1B). As predicted from the cloned sequences, primers F5 and R5 amplify a 200-bp fragment from the 3′ end of reverse transcribed C57BL/10 RNA and a 585-bp fragment from Balb/C genomic DNA. PCR amplification from other genomic DNA samples yields fragments of approximately 585 bp (class II) or 200 bp (class I). PCR products were resolved on a 4% agarose gel (3% NuSieve agarose, 1% LE agarose, FMC Bioproducts) with *Hae*III-digested φX174 DNA as a molecular weight standard.

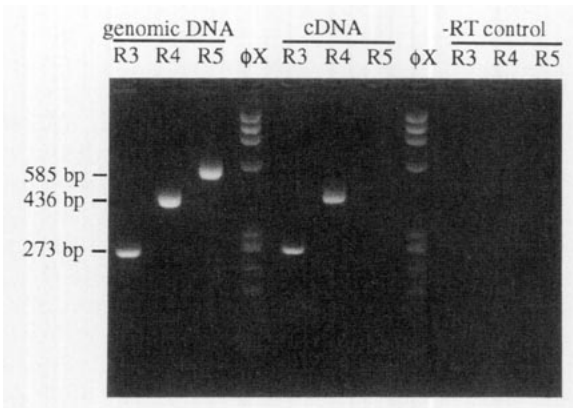

FIG. 4. Transcription of the 3′ end of Balb/C *Phk-γM* gene. **(Left)** PCR amplification of Balb/C genomic DNA using primer F5 and primers R3, R4, or R5 as indicated. **(Center)** PCR amplification of Balb/C reverse transcribed RNA using the same primers. **(Right)** PCR amplification of a mock reverse transcription reaction (Balb/C RNA incubated without reverse transcriptase). RNAs were treated with DNase I prior to reverse transcription. Gel analysis was as described in the legend to Fig. 3.

suggesting that the class II gene arose from the class I gene. The class I gene structure is present in many laboratory strains of mice, including C57BL/10 and ICR, as well as in *M. domesticus* and *M. spretus*. The class II gene structure was identified from the Balb/C gene sequence and is shared by *M. m. castaneus* and *M. m. molossinus*. *M. domesticus* is known to be more closely related to *M. m. castaneus* and *M. m. molossinus* than it is to *M. spretus* (Bonhomme and Guenet, 1989). The fact that *M. domesticus* and *M. spretus* share the class I gene structure also suggests that the class II allele is the derivative state and arose in the ancestor of *M. m. castaneus* and *M. m. molossinus* after their separation from *M. domesticus*. The common inbred laboratory strains of mice are thought to have arisen from crosses between *M. domesticus* and *M. m. castaneus* and/or *M. m. molossinus*, the latter being a naturally occurring hybrid of *M. m. musculus* and *M. m. castaneus* (Bonhomme and Guenet,

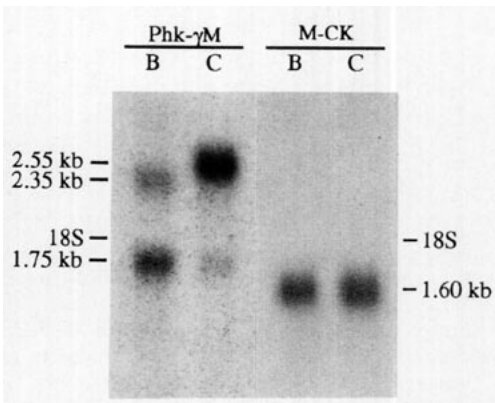

FIG. 5. Northern analysis of Phk-γM mRNA in Balb/C and C57BL/10 mice. Ten micrograms of total skeletal muscle RNA were loaded in each lane. After transfer of the RNA to Nytran membranes (Schleicher and Schuell), the membrane was sequentially hybridized with Phk-γM cDNA Phk-2 (Chamberlain *et al.*, 1987) and mouse muscle creatine kinase cDNA pMCK-36 (Jaynes *et al.*, 1986). The hybridized blot was exposed to X-ray film for 6 days **(Phk-γM)** or 4 h **(MCK)**.

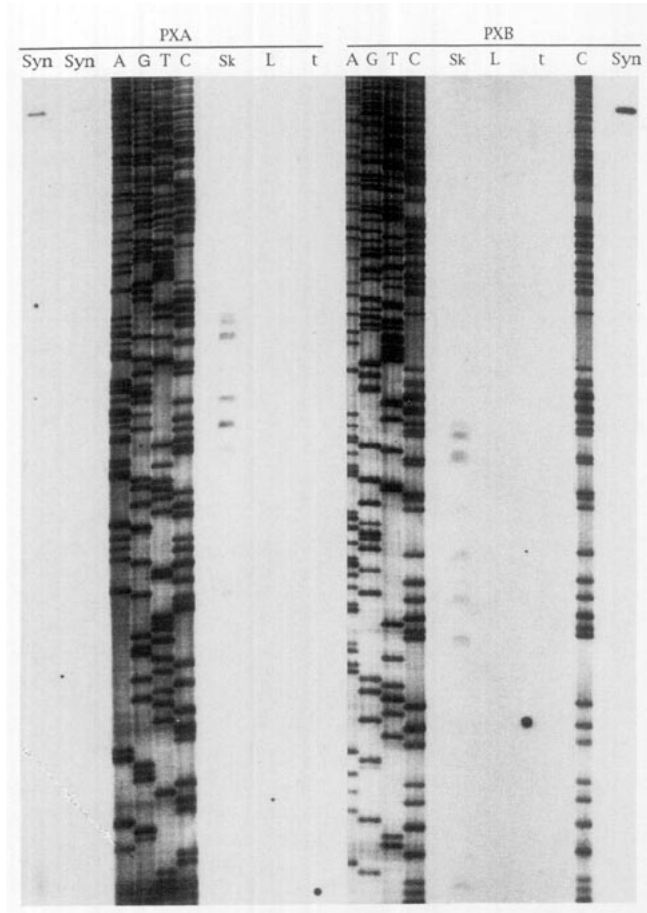

FIG. 6. Mapping the transcription start site by primer extension with primers PXA and PXB. Lanes labeled **Syn** contain an aliquot of a primer extension reaction that used the indicated primer and 25 pg synthetic sense-strand RNA from plasmid pPhkN. Lanes labeled **Sk, L,** and **t** contain 3/7 of the primer extension reactions that used the indicated primer and either 2 μg poly(A)$^+$ C57BL/10 skeletal RNA, 2 μg poly(A)$^+$ C57BL/10 liver RNA, or 3 μg tRNA. pPhkR was sequenced with the appropriate primer, either PXA or PXB, and electrophoresed in parallel with the primer extension reactions. The corresponding RNA sequence is read 5′ to 3′ beginning from each comigrating primer extension and proceeding toward the bottom of the gel. The **C** lane of the PXB sequencing reaction is shown as a size standard for the PXB synthetic RNA control.

1989). We conclude that ICR, C57BL/10 and at least four other strains of laboratory mice have inherited the class I *Phkg* structure from *M. domesticus,* whereas the Balb/C strain has inherited the class II structure from *M. m. molossinus* and/or *M. m. castaneus.*

The fact that Balb/C and *M. m. molossinus* share the same size STR in Δ2, rather than the shorter STR observed in *M. m. castaneus,* supports the hypothesis that *M. m. castaneus* genetic material entered the inbred laboratory strains via *M. m. molossinus* (Bonhomme and Guenet, 1989). It is possible, however, that *M. m. castaneus* and *M. m. molossinus* populations are polymorphic for the STR. CASA/RK has been propagated as an inbred strain and therefore would not display heterozygosity. One additional noninbred *M. m. castaneus* and several *M. spretus* samples could not be amplified using primers F5 and R5, suggesting that polymorphisms are present in the primer binding sites of these individuals.

Besides Phk-γM, several other cDNAs terminating in B2 repeat sequences have been reported (Ryskov *et al.,* 1983; Kress *et al.,* 1984). B2 sequences are approximately 190 bases long, contain an internal RNA polymerase III promoter, and end with the consensus polyadenylation signal AATAAA (Ryskov *et al.,* 1983). These elements are believed to replicate through reverse transcription of RNA intermediates (Jagadeeswaran *et al.,* 1981) and can integrate into a previously nicked or broken DNA molecule, creating a direct repeat at the insertion site (Deininger, 1989). Genes that encode mRNAs ending with a B2 repeat are likely to have existed prior to insertion of the B2 element, which raises the question of how the corresponding mRNAs were terminated prior to the insertion event. One answer is illustrated by sequence comparisons of three closely related members of the murine class I major histocompatibility (MHC) genes (Kress *et al.,* 1984). In the homologous D and L genes a B2 repeat provides the polyadenylation signals for the mRNAs. The closely related K gene does not carry this B2 insertion; transcripts from the K gene terminate at sequences that are retained, with minimal use, in the 3′ flanking region of the D and L genes.

The evolution of *Phkg* gene structure is both analogous to and more complicated than the MHC example since two B2 repeats, rather than one, have modified the 3′ end of *Phkg.* The Balb/C (class II) and C57BL/10 (class I) genes produce transcripts of 2.35 and 2.55 kb, respectively; these are colinear with the 1.75-kb transcripts produced in both strains but contain longer 3′ untranslated regions. There is no obvious need to produce alternate 3′ ends since all three mRNAs include the protein coding region, and the relative ratio of the 1.75- and 2.55-kb mRNAs is similar in all tissues and developmental stages of C57BL/10 mice (Chamberlain *et al.,* 1987 and unpublished observations). We hypothesize that the primordial gene for Phk-γM produced a single transcript terminating at the site used to produce the 1.75-kb mRNA. Insertion of a B2 repeat into the 3′ flanking region introduced a strong distal polyadenylation site, which is used preferentially in C57BL/10 and

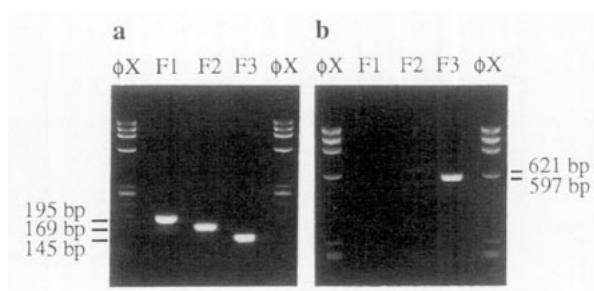

FIG. 7. Confirmation of the *Phkg* transcription start sites in Balb/C skeletal muscle by PCR. (**a**) PCR products amplified from Balb/C genomic DNA using the forward primers F1, F2, and F3 and exon 1 reverse primer R1. Specific amplification conditions were 60°C annealing and 1-min extensions for 35 cycles. (**b**) PCR amplification of reverse-transcribed RNA from Balb/C skeletal muscle, using the forward primers F1, F2, and F3 and exon 6 reverse primer R2. PCR conditions were as in (**a**) except that the annealing temperature for primers F1 and F2 were decreased to 57°C. Gel analysis was as described in the legend to Fig. 3.

ICR mice (class I) to produce the 2.55-kb transcript. A second, more proximal, B2 insertion into a class I gene produced the class II gene structure. This second B2 insertion introduced an intermediate polyadenylation site and, in Balb/C mice, allowed a resurgence in the use of the original upstream termination site. The upstream insertion in the class II genes, which contains two sequences duplicated and inverted from exon 10 of the class I genes (Fig. 1B), is likely to have arisen in conjunction with the B2 insertion as a result of chromosome breakage and repair. Evidence for this hypothesis comes from the structure of the two B2 repeats at the 3′ end of *Phkg*. Both are "full-length" B2 repeats flanked by perfect direct repeats and both retain a high degree of sequence identity with the consensus B2 sequence, suggesting that insertion of these elements has occurred recently in the evolution of this gene.

Phosphorylase kinase activity is detectable in many tissues including skeletal muscle, cardiac muscle, brain, and liver, as well as in leukocytes, erythrocytes, and fibroblasts (Picket-Gies and Walsh, 1986). In addition to the Phk-γM isoform, a testis-specific isoform Phk-γT has been isolated (Hanks, 1989). In rats, the two γ subunits display 59% amino acid sequence identity (Calalb *et al.*, 1992). It remains unclear which gene encodes the catalytic subunit of Phk in liver and hematopoetic cells; neither the testis nor the muscle γ subunit genes are expressed to an appreciable degree in liver (Fig. 6; Chamberlain *et al.*, 1987; Calalb *et al.*, 1992). Southern analysis experiments used to map the human homolog of *Phkg* to human chromosome 7 revealed the presence of cross-hybridizing sequences on human chromosomes 11 and 7 (Chamberlain *et al.*, 1987; da Cruz e Silva and Cohen, 1987). However, no cross-hybridizing sequences have been detected in mice with Phk-γM cDNA probes, even at low stringency (Chamberlain *et al.*, 1987; Bender and Emerson, 1987). Since the gene for Phk-γT was not detected by hybridization of genomic Southern blots with Phk-γM cDNA probes, a liver-specific isoform for the γ subunit of Phk may also display only moderate sequence identity with the known isoforms. Identification of all of the genes for the various isoforms of Phk would enable identification of the genetic defects in the many types of human Phk deficiencies and would allow analysis of the regulatory elements that control the tissue-specific expression patterns of the separate isoforms.

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