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Genetics

Isolation of cDNA clones for the catalytic γ subunit of mouse muscle phosphorylase kinase: Expression of mRNA in normal and mutant *Phk* mice

(sequence analysis/tissue-specific expression/chromosomal mapping)

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ABSTRACT **We have isolated and characterized cDNA clones for the γ subunit of mouse muscle phosphorylase kinase (γ-Phk). These clones were isolated from a λgt11 mouse muscle cDNA library via screening with a synthetic oligonucleotide probe corresponding to a portion of the rabbit γ-Phk amino acid sequence. The γ-Phk cDNA clones code for a 387-amino acid protein that shares 93% amino acid sequence identity with the corresponding rabbit amino acid sequence. RNA gel blot analysis reveals that the muscle γ-Phk probe hybridizes to two mRNA species (2.4 and 1.6 kilobases) in skeletal muscle, cardiac muscle, and brain, but does not hybridize to liver RNA. Phk-deficient I-strain (*Phk*) mouse muscle contains reduced levels of γ-Phk mRNA as compared with control mice. Although the *Phk* defect is an X-linked recessive trait, hybridization to a human–rodent somatic cell hybrid mapping panel shows that the γ-*Phk* gene is not located on the X chromosome. Rather, the γ-*Phk* cross-hybridizing human restriction fragments map to human chromosomes 7 (multiple) and 11 (single). Reduced γ-Phk mRNA in I-strain mice, therefore, appears to be a consequence of the *Phk*-mutant trait and does not stem from a mutant γ-subunit gene.**

Phosphorylase kinase (Phk; ATP:phosphorylase-*b* phosphotransferase, EC 2.7.1.38) is a crucial glycogenolytic regulatory enzyme in several animal tissues (for review see refs. 1 and 2). This enzyme undergoes complex regulation in response to both phosphorylation and intracellular calcium ion levels. The rabbit and mouse holoenzymes are composed of 16 subunit proteins containing equimolar ratios of 4 subunit types known as α, β, γ, and δ (3). Skeletal muscle contains the highest amounts of Phk enzymatic activity, although activity is also observed in liver, cardiac muscle, brain, and several other tissues (4). Kinetic differences exist between the cardiac and adult skeletal muscle enzymes, and the liver and skeletal muscle enzymes are known to be separate isozymes (5–7). Cardiac muscle and red skeletal muscle Phk contain an isoform of the α subunit, termed α′, distinct from that found in white skeletal muscle (8). However, it is not known whether separate isoforms of the catalytic γ subunit, the β subunit, or the δ subunit (calmodulin) exist.

Phk is defective or absent in a number of glycogen storage diseases of mice and humans. Of these the most common is a human X-linked recessive Phk deficiency that affects liver and hematopoietic cells, but not muscle (9). Human muscle Phk deficiency is rare and appears to be transmitted in an autosomal fashion (10). In addition, two mouse mutants with Phk defects have been characterized. The first, termed I-strain (*Phk*) mice, has an X-linked recessive disease that results in the presence of <0.2% of the normal levels of Phk in muscle whereas the liver enzyme is unaffected (11, 12). The second mutant, termed V-strain mice, contains reduced levels of Phk enzymatic activity in muscle, liver, and heart. This mutation is transmitted in an X-linked dominant fashion (13). While V-strain mice contain an abnormal Phk protein in affected tissues, I-strain mice display a virtual absence of the enzyme in skeletal muscle. The identity of the mutant gene in any of these defects is unknown, and study of the diseases has been hampered by the unavailability of cloned probes for the Phk subunits. We report here the isolation of cDNA clones for the γ subunit of mouse muscle Phk. We also present data on the patterns of expression of muscle γ-Phk mRNA in normal and I-strain (*Phk*) mice.

MATERIALS AND METHODS

A cDNA library was prepared in λgt11 using RNA isolated from adult C57BL/10 mouse muscle essentially as described (14, 15). The synthetic γ-Phk oligonucleotide probe TC12 was kindly provided by Joel Habener (Harvard University). cDNA clones corresponding to the muscle γ-*Phk* gene were identified by low stringency screening of the λgt11 library with the probe TC12. Prehybridization was in 6× SSC (1× SSC = 150 mM sodium chloride/15 mM sodium citrate, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution = 0.2% bovine serum albumin/0.2% Ficoll/0.2% polyvinylpyrrolidone), 50 mM sodium phosphate (pH 6.8), sonicated herring sperm DNA at 0.1 mg/ml, and 0.1% sodium pyrophosphate, overnight at 42°C. Hybridization was in the same solution but containing 5% (wt/vol) dextran sulfate, 1× Denhardt's solution, and labeled TC12 at 10^6 cpm/ml for 24 hr at 37°C. Hybridized filters were washed in 2× SSC containing 0.1% sodium pyrophosphate and 0.1% sarkosyl at room temperature. Oligomers were end-labeled with T4 polynucleotide kinase to a specific activity of 10^9 dpm/μg. Purified insert from one of the isolated cDNA clones (designated γ-Phk2) was nick-translated (16) and used to rescreen the cDNA library for clones with larger inserts. This latter hybridization was performed essentially as described above except that 5× SSC at 65°C was used and that after hybridization the filters were washed in 0.1× SSC at 65°C.

RNA for RNA gel blot analysis was prepared from various tissues of control (ICR) and *Phk* (I/st) mice by the guanidine isothiocyanate–CsCl method (17). RNA gel blot and Southern analyses were performed using standard protocols (18, 19). DNA sequencing was performed via the Sanger dideoxy method using M13mp18 and mp19 vectors (20, 21). The construction and analysis of human–rodent somatic cell hybrid DNAs have been described (22, 23). I-strain (I/st) mice were kindly provided by Vernon Chapman (Roswell Park Memorial Institute, Buffalo, NY).

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RESULTS

Isolation of cDNA Clones to γ-*Phk*. A mouse muscle cDNA library was constructed in the vector λgt11 for the purpose of isolating clones to γ-*Phk*. The library contained 1.5×10^6 independent recombinants, in a background containing 50% nonrecombinant phage. A synthetic 51-base oligonucleotide probe, designated TC12, was used to screen for γ-*Phk* sequences (Fig. 1). This probe was designed by selecting a region of the published rabbit protein sequence that contained amino acids with minimal codon redundancy (24). The region chosen was amino acids 240–256, and the probe was synthesized by using the most frequently occurring codon for any amino acid with redundant codons (25). Initial low-stringency screening of 2×10^5 recombinants resulted in the identification of four γ-Phk cDNA clones. None of these isolates contained full-length inserts, so the library was rescreened with the 600-base-pair insert from one of the clones, pγ-Phk2. This second screening resulted in 28 positively hybridizing plaques, from which the entire coding sequence of the γ-Phk mRNA was deduced. Fig. 1 shows the cDNA and predicted amino acid sequences and compares them to the TC12 oligomer and rabbit amino acid sequences. The oligomer shared 80% (41/51) homology with the isolated cDNAs, while the rabbit and mouse proteins share 93% amino acid sequence identity. All of the sequence shown in Fig. 1 was confirmed by analysis of between two and four independent isolates from the cDNA library.

```
CCC CCT GAG ATC CAC GTG ACC ATG ACC CGG GAT GAC GCC CTC CCT GAC TCG CAT TCT GCA
                        Met Thr Arg Asp Asp Ala Leu Pro Asp Ser His Ser Ala
                                                 Ala             Gly     Thr

CAG ACT TTC TAC GAG AAC TAT GAG CCC AAG GAG ATC CTG GGC AGG GGA GTC AGC AGT GTG
Gln Thr Phe Tyr Glu Asn Tyr Glu Pro Lys Glu Ile Leu Gly Arg Gly Val Ser Ser Val
His Gly

GTC AGG CGC TGC ATC CAC AAA CCC ACA TGC CAG GAA TAC GCA GTG AAA ATC ATT GAC ATC
Val Arg Arg Cys Ile His Lys Pro Thr Cys Gln Glu Tyr Ala Val Lys Ile Ile Asp Ile
                                        Lys                                 Val

ACC GGA GGA GGA AGC TTT AGC TCT GAG GAG GTA CAG GAG CTT CGG GAA GCC ACC CTG AAG
Thr Gly Gly Gly Ser Phe Ser Ser Glu Glu Val Gln Glu Leu Arg Glu Ala Thr Leu Lys
                         Ala

GAG GTG GAC ATC CTG CAG AAG GTC TCG GGA CAC CCC AAC ATC ATA CAG CTG AAG GAC ACT
Glu Val Asp Ile Leu Gln Lys Val Ser Gly His Pro Asn Ile Ile Gln Leu Lys Asp Thr
                    Arg

TAC GAG ACC AAC ACT TTC TTC TTC TTG GTA TTT GAT CTG ATG AAG AGA GGG GAA CTC TTT
Tyr Glu Thr Asn Thr Phe Phe Phe Leu Val Phe Asp Leu Met Lys Arg Gly Glu Leu Phe
                                                        Lys

GAC TAT CTC ACT GAG AAG GTC ACC TTA ACC GAG AAG GAA ACC AGA AAG ATC ATG CGG GCC
Asp Tyr Leu Thr Glu Lys Val Thr Leu Thr Glu Lys Glu Thr Arg Lys Ile Met Arg Ala
                                    Ser

CTG CTG GAG GTG ATC TGT ACC CTG CAC AAA CTC AAC ATT GTC CAT CGG GAC CTG AAG CCG
Leu Leu Glu Val Ile Cys Thr Leu His Lys Leu Asn Ile Val His Arg Asp Leu Lys Pro
                         Ala

GAG AAT ATC CTT TTG GAT GAC AAC ATG AAT ATA AAG CTC ACA GAC TTC GGG TTT TCC TGC
Glu Asn Ile Leu Leu Asp Asp Asn Met Asn Ile Lys Leu Thr Asp Phe Gly Phe Ser Cys
                             Asp

CAG CTG CAG CCA GGA GAG AAG CTC CGA GAG GTT TGT GGG ACT CCC AGT TAT CTG GCC CCT
Gln Leu Gln Pro Gly Glu Lys Leu Arg Glu Val Cys Gly Thr Pro Ser Tyr Leu Ala Pro
        Asp

GAA ATC ATA CAG TGC TCC ATG GAC GAC GGC CAT CCT GGC TAT GGG AAG GAG GTG GAC ATG
Glu Ile Ile Gln Cys Ser Met Asp Asp Gly His Pro Gly Tyr Gly Lys Glu Val Asp Met
            Glu                 Asn     Asn

TGG AGC ACA GGC GTC ATC ATG TAC ACT CTG CTG GCT GGC TCC CCG CCT TTC TGG CAC CGG
Trp Ser Thr Gly Val Ile Met Tyr Thr Leu Leu Ala Gly Ser Pro Pro Phe Trp His Arg

                        TC12: ATG ATC ATG TCT GGT AAC TAC CAG TTC GGT TCT CCT GAG
AAG CAA ATG CTG ATG TTG CGG ATG ATC ATG GAC GGC AAA TAC CAG TTT GGC TCA CCA GAG
Lys Gln Met Leu Met Leu Arg Met Ile Met Asp Gly Lys Tyr Gln Phe Gly Ser Pro Glu
                                        Ser     Asn
TGG GAC GAC TAC
TGG GAT GAC TAC TCT GAC ACA GTG AAA GAC TTG GTG TCT CGA TTC TTG GTG GTG CAA CCT
Trp Asp Asp Tyr Ser Asp Thr Val Lys Asp Leu Val Ser Arg Phe Leu Val Val Gln Pro

CAG GAC CGC TGC TCG GCG GAA GAG GCC TTG GCA CAC CCT TTC TTT CAG GAG TAC GTA GTA
Gln Asp Arg Cys Ser Ala Glu Glu Ala Leu Ala His Pro Phe Phe Gln Glu Tyr Val Val
     Lys       Tyr Thr                                                 Gln

GAA GAA GTA CGG CAC TTC AGC CCC CGA GGG AAG TTC AAG GTG ATC TGT CTA ACT GTG GTG
Glu Glu Val Arg His Phe Ser Pro Arg Gly Lys Phe Lys Val Ile Cys Leu Thr Val Val
                                                                        Leu

GCA TCA GTA AAG ATC TAC TAC CAG TAC CGT CGG GTG AAG CCG GTA ACC AGG GAG ATC GTC
Ala Ser Val Lys Ile Tyr Tyr Gln Tyr Arg Arg Val Lys Pro Val Thr Arg Glu Ile Val
                Arg

ATC CGA GAC CCC TAC GCC CTT CGG CCG CTG CGG AGG CTC ATT GAC GCC TAT GCT TTC CGT
Ile Arg Asp Pro Tyr Ala Leu Arg Pro Leu Arg Arg Leu Ile Asp Ala Tyr Ala Phe Arg

ATC TAC GGC CAC TGG GTG AAG AAA GGG CAA CAG CAG AAC AGA GCC GCC CTC TTC GAG AAC
Ile Tyr Gly His Trp Val Lys Lys Gly Gln Gln Gln Asn Arg Ala Ala Leu Phe Glu Asn

ACG CCC AAG GCT GTG CTC CTC TCC TTG GCT GAG GAG GAG GAC TTC TGA AGG GCA GCG GGG
Thr Pro Lys Ala Val Leu Leu Ser Leu Ala Glu Glu Glu Asp Phe End
                        Phe             ----    Asp     Tyr

GCC CAG GGG AAA GCA GAC AGA GGG ACT T
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FIG. 1. Sequence of the mouse muscle γ-Phk cDNA and predicted amino acid sequence of the protein. Also shown below the mouse protein sequence are amino acids of the rabbit γ-Phk sequence that differ from the mouse sequence (24). The 387-amino acid mouse enzyme shares 93% sequence identity with the rabbit subunit; amino acid 383 of the mouse enzyme is not present in the rabbit enzyme. The location and sequence of probe TC12 are also shown.

Analysis of the Mouse Muscle γ-*Phk* Gene. Muscle γ-*Phk* gene structure in control (C57BL/10) and mutant (I/st) mice was examined by Southern analysis (18). Five restriction endonucleases were used to digest liver DNA prepared from control and mutant mice, and Southern analysis was performed using nick-translated insert from pγ-Phk3 (bases 1–1004, Fig. 1). No differences were observed between the two mouse strains, suggesting that no gross abnormalities such as deletions or rearrangements were present in the I-strain mouse γ-*Phk* gene (data not shown). In addition, several of the digestions resulted in hybridization to single fragments, suggesting that the muscle γ-*Phk* gene might be present at a single copy per genome.

We next determined whether the muscle γ-*Phk* gene, like the *Phk* mutation, was X linked. Southern blots containing DNA from a human 49XXXXY cell line, a human 46XY cell line, and mouse male and female liver DNA were probed with the insert from pγ-Phk3. The results shown in Fig. 2 demonstrate that no X-chromosome dosage-dependent hybridization was obtained with the γ-Phk cDNA probes. Rehybridization of the blot with the human hprt cDNA probe pHPT31, which hybridizes with both the X-linked human and mouse *hprt* genes (26), resulted in the expected X-chromosome dosage-dependent hybridization (data shown for mouse digests only). These results suggest that the muscle γ-*Phk* gene is not on the X chromosome and, therefore, is not the site of the *Phk* mutation.

Definite assignment of the chromosomal location of the muscle γ-*Phk* gene required hybridization to a human–rodent somatic cell hybrid mapping panel. Twelve hybrid DNAs, as well as control human, mouse, and Chinese hamster DNAs, were digested with restriction endonucleases, Southern blotted, and hybridized to nick-translated insert from pγ-Phk3. Mouse γ-Phk cDNAs cross-hybridized with human DNA

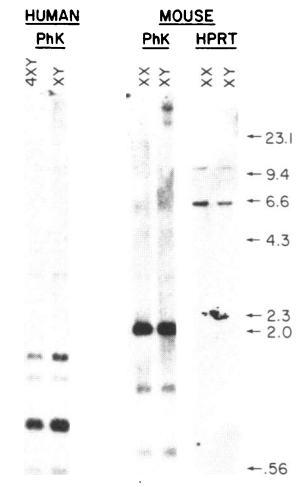

FIG. 2. X dosage-independent hybridization of pγ-Phk3. DNAs from a human 49XXXXY cell line (GM 1202b, 10 μg), a human 46XY cell line (RJK 859, 10 μg), and C57BL/10 mouse male and female liver (5 μg each) were examined by Southern analysis. DNA was digested with *Pst* I, electrophoresed on 0.7% agarose, transferred to nylon membranes, and hybridized to nick-translated pγ-Phk3 insert. Also shown is the result obtained upon rehybridization of the blot to the human hprt cDNA pHPT31 (data shown for mouse DNA only). Band intensities obtained upon autoradiographic exposure of the γ-Phk blot were independent of the number of X chromosomes in each DNA preparation. The result also demonstrates that mouse muscle γ-Phk cDNAs cross-hybridize with human DNA at high stringency [hybridization was carried out in 50% (vol/vol) formamide, 5× SSC at 42°C, and the blots were washed in 0.1× SSC at 55°C]. Molecular sizes in kilobase pairs (kbp) are shown.

when hybridized in 50% (vol/vol) formamide, 5× SSC at 42°C [no additional hybridization was obtained when the stringency was lowered by the use of 30% (vol/vol) formamide]. Four separate restriction enzymes were used on the mapping panel (*Eco*RI, *Xba* I, *Hin*dIII, and *Bam*HI), and similar results were obtained with each. The γ-Phk cDNA hybridized to four to seven human fragments with the various enzymes, and in each case one fragment mapped to human chromosome 11, whereas the remainder mapped to human chromosome 7 (Fig. 3, Table 1). These results again demonstrate that *γ-Phk* is not X linked, and hybridization observed on two chromosomes implies the presence of at least one human pseudogene or another gene with partial homology to *γ-Phk*. To distinguish the cross-hybridizing sequence from the functional structural gene, two of the hybrid cell line DNAs containing either human chromosome 7 or 11 were digested with *Hin*dIII, and a Southern blot was prepared and hybridized sequentially with different portions of the insert from cDNA pγ-Phk3. These two probes correspond to the 5′ and 3′ halves of pγ-Phk3 and are designated pγ-Phk9 (5′ half) and pγ-Phk2 (identical to the remaining portion of the pγ-Phk3 insert). As shown in Fig. 3, the two probes hybridized differentially to the chromosome 7 fragments, and only the pγ-Phk2 probe hybridized to the chromosome 11 fragment. These results suggest the chromosome 11 sequence is truncated, possibly a pseudogene, and that the functional structural gene resides on chromosome 7. These results are summarized in Table 1.

Expression of Muscle γ-Phk mRNA in Mouse Tissues. The tissue distribution of γ-Phk isoforms was compared between control (ICR) and mutant (I/st) mice. Total RNA was prepared from freshly excised tissue, electrophoresed through 1.5% agarose gels, and transferred to nitrocellulose (19). The RNA gel blots were hybridized to labeled insert from pγ-Phk3 at high stringency [50% (vol/vol) formamide, 5× SSC at 42°C], washed in 0.1× SSC at 60°C, and exposed to x-ray film. The results demonstrate that control and

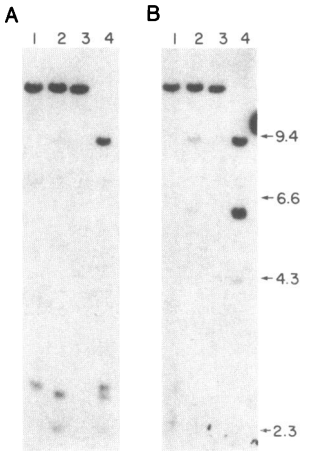

FIG. 3. Comparison of human chromosomes 7 and 11 hybridization patterns with mouse muscle γ-Phk cDNAs. Human–Chinese hamster somatic cell hybrid DNAs and control human and Chinese hamster DNAs were examined by Southern analysis as described in Fig. 2. The blot was sequentially hybridized to nick-translated inserts from either pγ-Phk2 (*A*) or pγ-Phk9 (*B*). Hybrid 1.11 contained human chromosomes 6, 8, 11, and X (lane 1, 10 μg), while hybrid MR 3.31 contained human chromosomes 7, 20, and X (lane 2, 10 μg). Lane 3 contained 10 μg of Chinese hamster DNA, while lane 4 contained 15 μg of human 46XY DNA. Additional Southern blot experiments (Table 1) indicate that only human chromosomes 7 and 11 hybridize to the probes. The results suggest that chromosome 11 contains a *γ-Phk* pseudogene, while the functional muscle *γ-Phk* gene is located on chromosome 7. The possibility of additional pseudogenes on chromosome 7 cannot be excluded by this data. Molecular sizes in kbp are shown.

I-strain mice contain two mRNA species that hybridize to the γ-Phk cDNA (Fig. 4). The major species in muscle and brain is 2.4 kilobases (kb) in size and accounts for approximately 80% of the observed hybridization signal, while the minor species is 1.6 kb long. Densitometric analysis reveals that I-strain skeletal muscle contains γ-Phk mRNA at a level 55% that of control mice, with both species of mRNA approximately equally reduced. This result was obtained by normalization of the data to the relative amount of hprt mRNA in each lane [Fig. 4, lanes 7 and 8, show the results of rehybridization of lanes 1 and 2 to the mouse hprt cDNA pHPT5 (27)]. Longer exposure of the autoradiograms reveals that neonatal skeletal muscle, adult heart, and adult brain contain small amounts of two γ-Phk mRNA species that comigrate with those from adult muscle (similar results were obtained with the corresponding I-strain tissues, data not shown). Ten-day-old mouse muscle also contained these same two hybridizing species, although at a level only slightly higher than in neonatal muscle, and no hybridization was obtained with adult liver, spleen, or kidney RNAs (data shown for liver only). These results show that γ-Phk mRNA is expressed in two forms in skeletal muscle, heart, and brain, and that a cDNA to muscle γ-Phk mRNA does not cross-hybridize with liver RNA at high stringency.

DISCUSSION

We have isolated and characterized cDNA clones to *γ-Phk*. These clones span the entire coding and portions of the noncoding regions of the γ-Phk mRNA. The amino acid sequence predicted from these cDNA clones is a 387-residue protein that shares 93% amino acid sequence identity with the corresponding rabbit subunit (Fig. 1) (24). Approximately 60% of the amino acid changes between the mouse and rabbit proteins are conservative substitutions.

Isolation of the muscle γ-Phk cDNA clones permitted analysis of possible involvement of this gene in the Phk-deficiency mutation of I-strain (*Phk*) mice (11). Several lines of evidence suggest involvement of *γ-Phk* in the X-linked I-strain mouse defect. Phk is known to be composed of four subunits, α, β, γ, and δ (1, 3), and the β and δ (calmodulin) subunits are encoded by autosomal genes (ref. 12; Anthony Means, personal communication). The presence of two α-subunit isoforms in skeletal muscle suggests that the α-subunit gene(s) may not contain the *Phk* mutation; however, this regulatory subunit has not been cloned, and it is not known on which chromosome the gene(s) are present (8, 12). It has been shown that the isolated γ subunit possesses considerable enzymatic activity both by itself and when complexed with the δ subunit calmodulin (28, 29). The observation that I-strain muscle contains <0.2% of the control levels of Phk activity argues that functional γ subunit is not present at an appreciable concentration (4, 6, 12). Furthermore, no Phk protein is detectable in I-strain skeletal muscle via standard purification protocols or immunoprecipitation techniques (12). These results suggest that the X-linked *Phk* defect might reside either within the structural gene for the γ subunit or in a regulatory gene that affects *γ-Phk* transcription, mRNA processing, translation, or post-translational protein modification.

To explore these possibilities we initially determined whether the γ-subunit gene was located on the X chromosome. Recombinant inbred mouse studies have indicated that the *Phk* mutation maps to within 1 cM (M = morgan) of the X-linked mouse muscular dystrophy mutation *mdx*, which may be equivalent to human Emery–Dreifuss muscular dystrophy (Vernon Chapman and Graham Bulfield, personal communication). Southern blot experiments demonstrated that γ-Phk cDNAs hybridized to mouse and human DNA in an X-chromosome-independent manner (Fig. 2). More direct

Table 1. Segregation of human γ-*Phk*-like sequences and human chromosomes in human–rodent hybrids

Cell hybrid	*Hin*dIII fragment*		Human chromosome																						
	A–C, E, F	D	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
1.11	−	+	−	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−
16.1	−	+	−	+	+	+	+	+	−	−	−	−	+	−	−	−	−	+	−	+	+	−	+	−	+	+
4.2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
TS3.2FAT	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−
MR5.11	+	+	+	+	+	−	−	−	+	−	+	+	+	−	+	−	−	+	−	+	−	−	+	+	+	−
6.1HZ	−	+	+	−	+	+	−	+	−	+	−	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+
8.2	+	+	−	+	+	+	−	−	+	+	−	+	+	+	−	+	+	+	−	−	−	+	−	−	+	−
MR1.21	+	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+	−
MH-74†	+	−	−	+	+	−	+	+	+	+	−	−	−	+	+	+	+	−	+	+	+	+	+	+	+	−
MH-18†	+	+	−	−	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+	−	−	+	+	−	−	−
SA-5†	+	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	+	−	+	−	−	+	−	−	−	−
MR3.31	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−
Discordant (%)	A–C, E, F		58	42	42	67	58	58	0	50	42	50	58	25	42	42	25	50	33	50	67	25	50	58	50	75
	D		33	33	17	25	50	33	58	25	25	25	0	50	33	50	50	25	58	42	42	50	25	50	42	33

Data shown is taken from Southern blots of *Hin*dIII digested hybrids (e.g., Fig. 3). Identical results were obtained with *Eco*RI, *Bam*HI, and *Xba* I. +, Presence of a hybridizing sequence or chromosome. −, Absence of a hybridizing sequence or chromosome.

*Six human *Hin*dIII fragments cross-hybridize with γ-Phk3 cDNA. These are designated: A, 9.4 kb; B, 6.0 kb; C, 4.3 kb; D, 2.95 kb; E, 2.8 kb; F, 2.4 kb. Fragments A–C, E, and F cosegregate and map to chromosome 7. Fragment D maps to chromosome 11. Fragments A, B, and C hybridize to γ-Phk9 cDNA, whereas fragments A, D, E, and F hybridize to γ-Phk2 cDNA (Fig. 3).

†MH-18 contains a translocation chromosome. It includes the long arm of 9 (9q12→qter) and the long arm of 17 (17q11→qter). MH-74 includes a ring 17 (p13.3q25.3). SA-5 is derived from fusion of Lmtk$^-$ × GM1139 and contains the translocation t(15;17)(15q15;p13).

‡Discordant means the presence of a hybridizing sequence and absence of a specific chromosome or vice versa. No discordance determined chromosome assignment.

evidence was provided by screening a human–rodent somatic cell hybrid mapping panel that contained various human chromosomes on a mouse or Chinese hamster background (22, 23). These studies demonstrated conclusively that mouse muscle γ-Phk cDNAs do not hybridize with the human X chromosome, but instead map to human chromosomes 7 and 11 (Table 1). Fig. 3 illustrates that chromosome 11 contains either a γ-*Phk* pseudogene or a partially homologous gene, while the functional structural gene is located on chromosome 7. This conclusion is drawn from the observation that separate regions of the γ-Phk cDNA show hybridization to genomic DNA fragments in the chromosome 7 containing hybrids, implying an extended intron containing gene structure; whereas only a portion of the γ-Phk cDNA probe hybridizes to a DNA fragment in chromosome 11 containing hybrids, suggesting a truncated pseudogene or another partially homologous gene. While only tentative conclusions can be drawn as to the chromosomal location of the muscle γ-*Phk* gene in mice, Ohno's law of conservation of X linkage between mammals argues that the gene will not be on the mouse X chromosome, in agreement with the mouse X-dosage data in Fig. 2 (30). Conservation of X linkage has been demonstrated with numerous cloned probes, and no exceptions to Ohno's law have been observed (31). These results prove that the γ-*Phk* gene is not X linked in mammals. Of the protein kinase genes mapped to human chromosomes, only the *met* oncogene is known to be located on chromosome 7 (32). That gene is within 1 cM of the cystic fibrosis gene, and speculation regarding a protein kinase role in cystic fibrosis warrants regional mapping of the γ-*Phk* gene on chromosome 7 (33, 34). It has been shown (24, 32) that v-src protein has homologies to both met and γ-Phk. As expected, γ-Phk and met are also homologous in the regions sharing homology with v-src; however, γ-Phk and v-src are more closely related to each other than either is to met (data not shown).

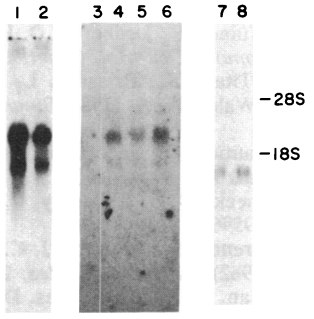

FIG. 4. RNA gel blot analysis of γ-Phk mRNAs. RNA isolated from various tissues of control (ICR) and mutant (I/st) mice was denatured with formaldehyde/formamide, electrophoresed through a 1.5% agarose/formaldehyde gel, and transferred to nitrocellulose (19). Blots were then hybridized to nick-translated pγ-Phk3 insert [50% (vol/vol) formamide, 5× SSC at 42°C], washed in 0.1× SSC at 60°C, and exposed to x-ray film. The blot was then stripped and rehybridized to nick-translated mouse hprt cDNA pHPT5. Lanes: 1, ICR adult skeletal muscle; 2, I/st adult skeletal muscle; 3, ICR liver; 4, ICR neonatal skeletal muscle; 5, ICR brain; 6, ICR cardiac muscle; 7, lane 1 rehybridized to hprt; 8, lane 2 rehybridized to hprt. Lanes 1, 2, 7, and 8 are from a 24-hr exposure of the blot to x-ray film; lanes 3–6 are from a 100-hr exposure. Densitometry of lanes 1 and 2 utilized a 6-hr exposure of the blot, while densitometry of lanes 7 and 8 was from the autoradiogram shown. Lanes 1, 2, and 4–8 contained 10 μg of total RNA, whereas lane 3 contained 3 μg of poly(A)$^+$ RNA.

Analysis of γ-Phk mRNA was carried out by hybridizing RNA isolated from various tissues of I-strain and control mice with γ-Phk cDNA probes. Two species of RNA hybridized to the γ-Phk probe at high stringency in neonatal and adult skeletal muscle, cardiac muscle, and brain (Fig. 4). No hybridization was observed with spleen or kidney RNA or with liver poly(A)$^+$ RNA. Since liver and muscle tissue contain high levels of Phk enzymatic activity, these results suggest that there are separate isoforms of γ-Phk in these tissues (1, 4). Hybridization to two mRNAs in muscle and brain raises the possibility that these tissues may contain two isoforms of γ-Phk, although it is equally possible that the RNAs simply differ in their polyadenylylation sites. In addition, Southern analysis suggests that muscle γ-Phk cDNAs hybridize to a single gene in mice at high and low stringency [for example, *Eco*RI, *Bam*HI, and several other restriction endonuclease digestions of C57BL/10 mouse

DNA result in probe hybridization to single bands (data not shown)]. This result suggests that the two RNA species observed on RNA gel blots may be transcribed from a single gene (Fig. 4). Control and I-strain mouse skeletal muscle contain essentially the same mRNAs although in different quantities (Fig. 4). The two hybridizing mRNAs are present in I-strain muscle at ≈55% of the levels observed in control mice when the data is normalized to the relative level of hprt mRNA in each lane. Although this difference in mRNA levels is significant and reproducible, it is not sufficient to explain the virtual lack of Phk activity in I-strain muscle.

γ-Phk may require association with the other Phk subunits to acquire enzymatic activity, which is known to be retained upon dissociation of the subunits (28, 29). A mutant α-subunit gene (whose chromosomal location is unknown) could, therefore, be responsible for the I-strain mouse phenotype. Alternatively, the Phk mutation may reside in a regulatory gene that is required for posttranslational processing of γ-Phk or for processing of the γ-subunit mRNA. Improper mRNA processing could lead to a lack of γ-Phk by virtue of the mRNA being nontranslatable or by failure of the mRNA to encode a functional protein. Further experiments involving transfection of γ-Phk cDNA containing expression vectors into I-strain mouse myoblasts combined with S1 nuclease analysis should resolve the question of whether γ-Phk mRNA is functional in I-strain skeletal muscle.

In addition to the almost complete lack of Phk protein or activity in I-strain skeletal muscle, there is a partial reduction of Phk activity in I-strain cardiac muscle and brain when compared to control mice (4, 6). It is, therefore, of interest that cardiac muscle and brain also contain hybridizable γ-Phk mRNAs the same size as found in skeletal muscle. Partial reduction in enzymatic activity could be explained by the presence of multiple Phk isoforms in cardiac muscle and brain. Reduced enzymatic activity would, therefore, arise from a lack of the skeletal muscle isoform of Phk, with the residual activity being contributed by a brain and/or cardiac isoform. There is considerable precedence for separate cardiac and skeletal muscle isoforms of proteins being present at various levels in both muscle types, examples of which include creatine kinase and α-actin (14, 35, 36). Evidence supporting this possibility has been demonstrated (6) by the observation that cardiac muscle Phk has properties similar to neonatal skeletal muscle Phk, which are distinct from the properties of the adult skeletal muscle isoform. It has also been shown that Phk activity in neonatal I-strain skeletal muscle is not reduced to nearly the extent found in adult I-strain skeletal muscle (4, 6). The γ-Phk mRNA species observed in cardiac muscle and brain (Fig. 4) may, therefore, be skeletal muscle γ-Phk mRNAs that do not contribute to the Phk activity in I-strain mice, although cross-hybridization with presumptive cardiac and/or brain isoforms cannot be excluded.

We have examined the chromosomal location and tissue-specific expression of the mouse muscle *γ-Phk* gene. Failure of γ-Phk cDNA clones to hybridize to mammalian X chromosomes demonstrates that this gene is distinct from the mutant X-linked gene in I-strain mice. However, observation of reduced γ-subunit mRNA in I-strain skeletal muscle raises the possibility that the *Phk* mutation may reside within a regulatory gene that affects proper expression of γ-Phk mRNA.

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