A cDNA encoding the skeletal muscle phosphorylase kinase catalytic subunit \( \gamma \) has been isolated and sequenced. It contains 57 nucleotides of 5' nontranslated sequence, the entire coding sequence, and 1004 nucleotides of 3' nontranslated sequence. Probes derived from this \( \gamma \)-cDNA were used to investigate the expression of \( \gamma \)-catalytic subunit messenger RNAs (mRNAs) in liver, heart, and skeletal muscle tissues. The results demonstrate that the \( \gamma \)-mRNAs expressed in heart tissue are homologous to the skeletal muscle \( \gamma \)-mRNAs. However, in liver tissue, no homologous \( \gamma \)-mRNAs were detected. The implications of these results for understanding \( \gamma \)-isoform expression and the possibility of a liver-specific \( \gamma \)-gene are discussed.

The phosphorylase kinase enzyme is expressed primarily in liver, cardiac, and skeletal muscle tissues where it functions to couple glycogenolysis to hormonal stimuli (1, 2). Phosphorylase kinase isolated from each of these tissue sources is reported to have the same subunit structure, consisting of three regulatory subunits (\( \alpha, \beta, \) and calmodulin) and a catalytic subunit (\( \gamma \)) (3, 4). Only for the \( \alpha \)-subunit is there a known isoform. This isoform is designated \( \alpha' \) and replaces \( \alpha \) in slow-twitch muscle fibers and cardiac tissues (5-7). For the remaining subunits, no isoforms are known, yet the phosphorylase kinase enzymes isolated from each tissue source have different functional properties. For example, each enzyme contains calmodulin as an integral subunit, yet their calcium requirements are different (6, 8). Similarly, the activation of the heart and skeletal muscle phosphorylase kinase through the phosphorylation of the \( \alpha/\alpha' \, \beta \) and \( \beta \)-subunits results in a shift in the pH activity ratio 6.8:8.2 (6, 9); the liver enzyme, although activated by the phosphorylation of its \( \alpha \)- and \( \beta \)-subunits, does not exhibit this shift (8, 10, 11). These biochemical differences provide the basis for suggesting that a distinct phosphorylase kinase isoform is expressed in each tissue. Presumably, the molecular basis for these phosphorylase kinase isoforms is the expression of one or more subunit isoforms.

From the observations of heritable phosphorylase kinase deficiencies, it is evident that the expression of the phosphorylase kinase isoforms is subject to tissue-specific regulation. For example, in the \( \text{I/Lyn} \) mouse strain, an X-linked mutation results in a phosphorylase kinase deficiency in only its adult skeletal muscle (12). The liver and heart tissues express normal enzyme activity. In contrast, an autosomal mutation in the \( \text{gsd/gsd} \) rat strain results in a phosphorylase kinase deficiency in only the liver tissue, while the skeletal muscle and heart have normal enzyme activity (13). The phenotypes of these mutations demonstrate that independent mechanisms are involved in the expression of the liver and skeletal muscle phosphorylase kinase isoforms. These mechanisms could involve regulatory genes controlling the tissue-specific expression of the phosphorylase kinase enzyme, or they could involve the tissue-specific expression of a multigene family encoding one of the phosphorylase kinase subunits. The latter possibility implies that at least one of the phosphorylase kinase subunits is represented by distinct isoforms in skeletal muscle, heart, and liver.

The absence of previous reports of phosphorylase kinase subunit isoforms, other than \( \alpha \) and \( \alpha' \), may reflect the difficulty of obtaining sufficient material from heart and liver for detailed physical analysis. Generally, one-dimensional gel electrophoresis in the presence of sodium dodecyl sulfate has been used to identify the subunits solely on the basis of molecular weight. The more discriminating technique of isoelectric focusing has not yet been applied successfully to the analysis of the heart and liver phosphorylase kinase subunits. A different approach for identifying subunit isoforms is to use nucleic acid probes to compare the homology between the subunit messenger RNAs expressed in skeletal muscle, heart, and liver. This approach circumvents the problem of insufficient material and allows us to investigate the tissue-specific expression of phosphorylase kinase isoforms as exemplified by the \( \text{I/Lyn} \) and \( \text{gsd/gsd} \) mutations.

The \( \gamma \)-subunit was selected for investigation because it contains the domains necessary for binding the calmodulin subunit as well as the catalytic domain (14, 15). Isoforms of \( \gamma \) with differences in their calmodulin binding domains might account for the differences in calcium dependence between the liver, heart, and skeletal muscle isoforms.

To investigate the question of \( \gamma \)-isoforms and the possible genetic mechanisms regulating their expression, we have isolated a \( \gamma \)-encoding cDNA from mouse skeletal muscle RNA. This cDNA is almost full length, containing 57 nucleotides of 5' nontranslated sequence, the entire coding sequence for \( \gamma \), and 1004 nucleotides of 3' nontranslated sequence. Using this \( \gamma \)-cDNA as a hybridization probe, we have analyzed the expression of homologous RNAs in liver, heart, and skeletal muscle. The results demonstrate that in heart tissue \( \gamma \)-messages homologous to those in skeletal muscle are expressed at levels which can account for all of the \( \gamma \)-protein in heart tissue. We conclude that most, if not all, of the heart phosphorylase kinase enzyme contains the same \( \gamma \)-subunit as does...
skeletal muscle. In liver tissues, a message homologous to the skeletal muscle γ-cDNA was not detected. Considering the conditions used for detecting cross-hybridization, we infer that the liver γ-subunit is encoded by a gene distinct from that encoding the skeletal muscle and heart γ-subunit.

**EXPERIMENTAL PROCEDURES**

**Preparation of γ-cDNA—**RNA from mouse (ICR Swiss White) skeletal muscle tissue was chromatographed on oligo(dT)-cellulose (Collaborative Research, type 3) for the selection of the poly(A)+ RNA fraction. From this fraction, cDNA was prepared and cloned into the Agt11 vector according to published procedures (16, 17). The resulting cDNA library contains ≥8 × 10^6 independent recombinants. After amplification, the library contained 16% non-recombinants.

The amino acid sequence of the rabbit skeletal muscle γ-subunit was used for the identification of recombinants containing γ-cDNA inserts (18). From this sequence data, two stretches of five and seven amino acids were identified which, when translated to their triplet code, were acceptable for the preparation of synthetic oligonucleotides. These sequences are:

1. SaPhe Tyr Glu Asp Tyr Glu Pro
2. UUAU GAG AAU UAUA GAAG CCX
3. GlyU Glu Trp Asp Asp Tyr Pol

Two samples of mixed-batch deoxynucleotides were synthesized. Each sample contained ssDNA sequences complementary to all of the possible RNA sequences encoding each of the amino acid sequences. These synthetic oligonucleotides were synthesized by Mark Sepanski at the Carnegie Institute in Baltimore.

Both synthetic oligonucleotide samples were labeled with ^32P and used for plaque hybridization to plates containing 50,000 recombinant plaque-forming units (16). The hybridization procedure used tetramethylammonium chloride in the wash buffers as an A-T stabilizing agent (19). Hybridizing plaques were picked and rescreened until the poly(A)+ RNA fraction. From this fraction, cDNA was prepared and cloned into the Xgt11 vector according to published procedures (16, 17). The resulting cDNA library contains ≥8 × 10^6 independent recombinants. After amplification, the library contained 16% non-recombinants.

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**Fig. 1.** The nucleic acid sequence of the γ-cDNA is illustrated with the encoded amino acid sequence written above each line in single letter code. The amino acid differences between this mouse γ-subunit and the rabbit γ-subunit are written below each line of coding sequence. The X at amino acid 384 is for alignment and represents the absence of a corresponding amino acid in the rabbit γ-sequence. Rabbit γ is one amino acid shorter than the mouse γ-subunit. The start and stop codons are underlined as well as the poly(A) addition consensus sequences in the 3′ nontranslated region. The nucleic acid sequence shown has been confirmed by sequencing both strands of the γ-cDNA (see "Experimental Procedures").
Comparison of its open reading frame with the published rabbit skeletal muscle γ-protein sequence (18) identified it as a cDNA encoding the γ-subunit of phosphorylase kinase.

Fig. 1 shows the complete nucleotide sequence of this γ-cDNA, the translation of its open reading frame, and the amino acid differences between the encoded mouse skeletal muscle protein and that reported for rabbit skeletal muscle γ. The amino acid sequences of these two proteins are 93% homologous. Sixteen of the 27 substitutions are conservative. Of the remaining 11, none are in positions known to be conserved between the rabbit γ-subunit and other serine kinases (28). However, the mouse γ-protein has an additional glutamic acid near its carboxyl terminus (amino acid 384) that is absent from the rabbit protein sequence. This additional amino acid lies carboxyl-terminal to the putative calmodulin binding site (29, 30), and it is not clear whether this amino acid insertion has a particular significance to γ function.

The γ-cDNA sequence in Fig. 1 contains 57 nucleotides of 5' nontranslated sequence and 1004 nucleotides of 3' nontranslated sequence. The cDNA ends with the poly(A) addition consensus sequence AATAAA. Within the 3' nontranslated sequence, this consensus sequence is repeated four times between nucleotides 1558 and 1579. The possibility that these sequences are used as alternative poly(A) addition sites is not indicated by the S1 protection assay discussed later in this text.

The size and relative abundance of the γ-related RNAs expressed in liver, cardiac, and skeletal muscle tissues were analyzed by RNA blotting (Fig. 2). Poly(A)-containing RNA from each tissue was electrophoresed on denaturing agarose gels, blotted onto nitrocellulose, and hybridized with a 32P-labeled complementary RNA probe (24) spanning the entire coding region of the γ-cDNA. In skeletal muscle RNA, the major species of γ-related RNA is approximately 2500 bases in length. A minor species with an apparent length of 1900 bases also is evident. Both of these species are also present in the RNA sample from heart tissue, but neither is evident in the RNA sample from liver tissue under these conditions. The absence of cross-hybridizing γ-mRNAs in liver does not reflect a decreased abundance of the liver γ-message because in heart, at the same stringency, γ-mRNAs are detected, and it is estimated that phosphorylase kinase is almost equally abundant in heart and liver (31, 32). Thus, if the abundance of the γ-message reflects the abundance of its protein, it would be detectable in the liver RNA sample. We conclude that the absence of cross-hybridizing γ-mRNAs in liver tissue, under high stringency conditions, represents heterogeneity between the liver γ-mRNAs and the skeletal muscle γ-probe.

Under less stringent conditions for hybridization (defined under “Experimental Procedures”), several bands are evident in the liver RNA sample. Some of these bands have the same mobility as the two γ-messages noted in the skeletal muscle and heart RNA samples. However, the bands in the liver sample decrease in intensity as the conditions for washing increase in stringency. The loss of hybridization at increased stringency indicates that the homology of these bands with the skeletal muscle γ-probe is low. As a consequence, it is difficult to determine whether they represent the liver γ-messages or other related sequences. Nonetheless, from the high stringency conditions, we know that the liver γ-message is heterogeneous with that in skeletal muscle and heart.

S1 nuclease protection analysis was used to determine the extent of homology between the γ-mRNAs in heart and skeletal muscle. The S1 nuclease protection assay also was used to verify the absence of cross-hybridizing γ-mRNAs in liver tissue. Three probes were used in the S1 nuclease analysis. The first is complementary to the coding sequence of the γ-cDNA from the first XmaI site to nucleotide 1237. The other two probes are complementary to regions in the 3' nontranslated sequence from the XbaI site (1338) to the 3' end and from the second XmaI site (1449) to the 3' end. These two 3' probes were used in this assay to confirm the location of the sites where S1 nuclease protection stops and to control for the possibility that star activity of either restriction enzyme could artifactualy generate distinct fragments. All three probes were labeled at their 3' terminus and contain at their 5' ends sequences from the pEMBL plasmid they were subcloned in. Consequently, fragments protected by RNA will be distinguished from a self-annealed probe.

The results of the S1 nuclease analysis are shown in Fig. 3 using heart, liver, and skeletal muscle RNA. The probe complementary to the coding sequence is protected with RNA from either skeletal muscle or heart. The length of the protected fragment corresponds to full protection from the first XmaI site to nucleotide 1237. As predicted, this probe is not protected by the liver RNA sample, and neither are the probes complementary to the 3' segment of the γ-cDNA.

With the skeletal muscle and heart RNA samples, two major protected fragments are generated from both of the 3' probes. The longer fragment corresponds to full length protection from the XbaI (or XmaI) site to the 3' terminus of the γ-cDNA. This fragment is probably protected by the same γ-mRNA species that the γ-cDNA was cloned from. The shorter fragment corresponds to protection from the XbaI (or XmaI) site to nucleotide 2070 (±10 bases). This fragment is protected by a second γ-mRNA species which is identical in sequence with the γ-probe up to approximately nucleotide 2070 and is then significantly different in its remaining 3' sequence. These results indicate that there are primarily two
**Fig. 3.** S1 nuclease analysis. In A, the results of a S1 nuclease protection assay are shown. Three end-labeled probes, derived from the γ-cDNA, were used for solution hybridization to RNA from liver, heart, and skeletal muscle. After S1 nuclease digestion, the protected fragments were fractionated by gel electrophoresis and visualized by autoradiography. In adjacent wells, labeled standards of known length were electrophoresed, and their migration was used to estimate the length of the protected fragments. In B, these results are illustrated with respect to the γ-cDNA. The thatched regions on each probe represent plasmid sequences which must be digested to give a protected fragment (note in A some self-annealed and protected probe is indicated). The stars denote the end of the probe labeled with 32P. Refer to Fig. 1 for more detailed alignment of the protected fragments.
γ-mRNAs in heart and skeletal muscle and that there γ-mRNAs are homologous until nucleotide 2070. Because of this homology, we conclude that both γ-mRNAs are transcribed from the same gene. Their heterogeneity in sequence 3' from nucleotide 2070 probably results from alternative exon selection because no polyadenylation consensus sequence exists at an appropriate distance 5' to the region of heterogeneity.

From the S1 protection assay, there is no evidence of a fragment corresponding to protection by a γ-message using the internal polyadenylation consensus sequences between nucleotides 1558 and 1579. However, a third fragment corresponding to protection from the Xbal site to nucleotide 1710 is evident when the S1 nuclease digestion are done at 31°C. This fragment is not evident when the S1 nuclease digestion is done at 18°C with either of the 3' probes (results with the 3' Xmal probe are shown at 18°C). Because the sequence around nucleotide 1710 consists of a stretch of 12 (T)s and the fragment is only generated at the elevated S1 digestion temperature, we conclude that the fragment is an artifact resulting from the breathing of the r(U):d(A) region in the RNA:DNA hybrid during S1 nuclease digestion.

The results of the S1 nuclease protection assay indicate that there are two γ-mRNA species in heart and skeletal muscle. Both are homologous through their coding and 3' sequences until nucleotide 2070. From this experiment, we cannot determine the length of each message. However, we know that one γ-message must be at least 2225 nucleotides and the other at least 2070. These minimum estimates do not include any additional 3' and 5' nontranslated sequences that are outside of the γ-cDNA nor any of the poly(A)+ tail. These results, when compared with the Northern blots in Fig. 2, indicate that the γ-messages detected in the S1 nuclease experiment are too long to correspond to the band at 1900 nucleotides in the Northern blot. Presumably, the two messages detected in the S1 nuclease assay correspond to a doublet, seen on the Northern blot as a band of approximately 2500 nucleotides in length. If this interpretation is correct, then the 1900-nucleotide band represents either a third γ-mRNA species which is heterogeneous to the γ-probes in most of its 3' nontranslated sequence or it is a transcript from a gene more closely related to the skeletal muscle γ-gene than the liver γ-gene. If this band is a third γ-mRNA, we cannot rule out the possibility that it also has minor differences in its coding sequence with the γ-cDNA.

The relative abundance of the γ-mRNA in heart tissue can be estimated from the density of the protected fragments on the autoradiographs in Fig. 3. From these data, it is estimated that heart tissue contains approximately 10% of the γ-mRNA expressed in skeletal muscle. Estimates of the relative abundance of the phosphorylase kinase enzyme in heart are 5-10% of that in skeletal muscle (31, 32). Thus, the γ-mRNAs detected by the S1 nuclease assay are sufficient to account for all of the γ-protein expressed in heart.

DISCUSSION

The successful cloning of a mouse skeletal muscle γ-encoding cDNA has enabled us to investigate the expression of γ-messages in liver, heart, and skeletal muscle. From this investigation, we have gained an understanding of the tissue-specific expression of γ-isofoms and the molecular basis of phosphorylase kinase deficiencies. Furthermore, from the sequence of the mouse γ-cDNA, the amino acid sequence of the mouse skeletal muscle γ-subunit was determined and compared to that reported for the rabbit γ-subunit.

The amino acid sequence of the mouse γ-protein is 98% homologous with the rabbit γ-protein. The extent of homology between proteins from species not in the same phylogenetic order indicates that γ function requires conservation throughout most of its sequence. It was surprising therefore that the skeletal muscle γ-cDNA coding probe did not detect a cross-hybridizing message in liver tissue. Considering that a uniformly labeled ribo-oligonucleotide probe was used for hybridization to the Northern blots in Fig. 2, the absence of cross-hybridization indicates that the heterogeneity between the γ-messages in liver and skeletal muscle must be dispersed throughout their coding sequence. The most likely explanation of this result is that the liver γ-subunit and the skeletal muscle γ-subunit are encoded by separate genes. The divergence of these two genes should result in differences in the amino acid sequences of the two γ-proteins. Knowing these amino acid differences would help us to understand the structural basis for γ-isofoms and the contribution of these isofoms to the differences in calcium dependence and pH optima between the liver and skeletal muscle phosphorylase kinase isozymes. Toward this goal, we are attempting to clone and sequence the liver γ-mRNA.

Unlike liver, in heart, γ-mRNAs were detected homologous to the skeletal muscle γ-probe. The results of the S1 nuclease protection assay, where both the coding sequence probe and the 3' nontranslated sequence probes were protected by the RNA from both skeletal muscle and heart, indicates that the γ-mRNAs in both tissues are identical. Therefore, we conclude that the same γ-gene is expressed in both tissues, and thus the γ-subunits in heart and skeletal muscle are identical.

This conclusion has important implications for our understanding of the molecular basis for phosphorylase kinase isozyme expression. Although the calcium requirement of the heart phosphorylase kinase differs from that of the skeletal muscle enzyme, the results presented here demonstrate that the skeletal muscle γ-mRNA is expressed in heart at levels which can account for all of the γ-subunit expressed. This finding indicates that the difference in calcium dependence cannot result from differences in γ-isofoms unless these isofoms arise from post-translational modifications. For the skeletal muscle γ-subunit, no post-translational modifications were reported from the amino acid sequencing of the rabbit γ-subunit (18). However, the heart γ-subunit has not been analyzed in sufficient detail to eliminate the possibility of a tissue-specific post-translational modification of this subunit. Such modifications could generate a γ-isofom which affects the calcium dependence of the heart phosphorylase kinase isozyme. The alternative to invoking post-translational modifications of γ is the possibility that there are heart-specific isofoms of α' and/or β which affect the calcium dependence of the heart phosphorylase kinase enzyme. This latter possibility is supported by the finding that monoclonal antibodies to the α- and β-subunits affect the calcium dependence of skeletal muscle phosphorylase kinase (33, 34). This demonstrates that allosteric interactions, mediated by the α- and β-subunits, can affect the calcium requirement of phosphorylase kinase. Presumably, similar effects could result from the expression of α- and/or β-isofoms.

Tissue-specific isofoms of α and β (other than the α') have not yet been reported. The analysis of these subunits has been primarily limited to the skeletal muscle subunits. The corresponding liver and heart subunits have not been analyzed in sufficient detail for us to know whether the liver α is identical with the fast-twitch skeletal muscle α or whether the cardiac α' is identical with the slow-twitch skeletal muscle α'. The investigation of additional α/α' and β-isofoms is important for understanding the tissue-specific regulation of phosphorylase kinase expression and the biochemical differences between the phosphorylase kinase isozymes. Toward this goal,
we have begun to clone $\alpha$- and $\beta$-encoding cDNAs from skeletal muscle. These cDNAs can be used to compare the homologies between the corresponding messages in liver, heart, and skeletal muscle by techniques similar to those used for the analysis of the $\gamma$-messages.

The results of the S1 nuclease and Northern blot hybridizations indicate that the same $\gamma$-gene is expressed in both heart and skeletal muscle, while a different $\gamma$-gene is expressed in liver. These results provide insight for understanding the glycogen storage diseases (VIII) involving phosphorylase kinase deficiencies (35, 36). For example, in those disorders where there is a liver phosphorylase kinase deficiency, one possible explanation is a mutation in the liver $\gamma$-gene which prevents the expression of a functional $\gamma$-subunit in liver. In this case, the heart and skeletal muscle would be normal because they express a different $\gamma$-gene. This is a plausible explanation for the gsd/gsd rat phenotype. In contrast, in those disorders where a mutation results in only a skeletal muscle phosphorylase kinase deficiency, such as the I/Lyn mouse strain, the site of the mutation cannot be the skeletal muscle $\gamma$-gene, because, if it were, heart would exhibit the same deficiency. Therefore, alternative loci for the I/Lyn mutation must be considered.

Two possible loci for the X-linked I/Lyn mutation are a regulatory gene controlling the tissue-specific expression of the phosphorylase kinase enzyme (37), or a structural gene encoding the $\alpha/\alpha'$-subunits. The gene(s) encoding the skeletal muscle $\beta$-subunit are known to be autosomal and thus cannot be the site of the I/Lyn mutation. Although the possibility of a regulatory mutation has been discussed by Cohen (see Ref. 37), the possibility of a mutation in a skeletal muscle-specific $\alpha/\alpha'$-gene has not been discussed previously in the literature. This possibility requires that the $\alpha$- and $\alpha'$-subunits be encoded on the same gene because both fast-twitch and slow-twitch muscle fibers are deficient in phosphorylase kinase. Furthermore, it requires independent mechanisms for the regulation of $\alpha$ and $\alpha'$ expression in liver and heart versus skeletal muscle because only the skeletal muscle is affected. Thus, a single gene encoding both $\alpha$ and $\alpha'$ would contain at least two regulatory sequences; one mediating the tissue-specific expression in heart and liver versus skeletal muscle and another mediating the $\alpha$- versus $\alpha'$-isoform expression. The investigation of an $\alpha/\alpha'$-gene on the nucleic acid level will require an $\alpha$-encoding cDNA to determine the homology between the $\alpha$- and $\alpha'$-messages and to determine whether these messages map to the same gene or two different genes.

Further investigation of phosphorylase kinase isoforms and the molecular basis of phosphorylase kinase deficiencies will require the preparation of the $\alpha$- and liver $\gamma$-cDNAs. These we have begun to clone. They can be used to characterize the tissue-specific expression of their respective messages and to determine whether their genes map to either the I/Lyn or gsd/gsd mutations. Depending on these results, the possibility of a regulatory gene controlling the tissue-specific expression of one or more of the phosphorylase kinase subunits can be re-evaluated.

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REFERENCES