Volume 220, number 1, 36–42 FEB 04940 August 1987

Isolation and sequence analysis of a cDNA clone encoding the entire catalytic subunit of phosphorylase kinase

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Received 1 June 1987

Synthetic oligonucleotides have been used to isolate a 1.85 kb clone containing the full length coding sequence for the catalytic subunit of rabbit skeletal muscle phosphorylase kinase from a cDNA library constructed in λgt10. Sequence analysis of the clone predicted an amino acid sequence in agreement with a published primary structure. Inspection of the codon usage revealed a strong preference for G or C nucleotides at the third codon position as found for several other skeletal muscle proteins. This cDNA clone should facilitate identification of functional domains, including the calmodulin-binding site, and investigation of the molecular basis of X-linked phosphorylase kinase deficiencies.

Phosphroylase kinase; cDNA sequence; Calmodulin; X-linkage; Codon usage bias

1. INTRODUCTION

Phosphorylase kinase plays a central role in the regulation of glycogen metabolism. In response to both neuronal and hormonal stimuli the enzyme activates glycogen phosphorylase and inactivates glycogen synthase by phosphorylation, causing a net increase in glycogenolysis [1]. Rabbit skeletal muscle phosphorylase kinase (1300 kDa) has the structure $(\alpha\beta\gamma\delta)_4$, where α (145 kDa) and β (128 kDa) are regulatory subunits phosphorylated in response to adrenalin, γ (45 kDa) is the catalytic subunit [2], and δ is identical to calmodulin (17 kDa) conferring calcium dependency on the enzyme [3].

The amino acid sequence of the γ-subunit has been determined [4]. Alignment with the catalytic subunits of other protein kinases suggests that lysyl residue 48 interacts with the terminal phosphate of the substrate ATP. However, the sites that interact with phosphorylase and glycogen synthase and with the α- and γ-subunits [5] have not yet been identified. Two regions $\gamma_{322-345}$ [6] and $\gamma_{342-362}$ [7] have been proposed as the binding sites for calmodulin, but which is correct, if either, is unknown. Calmodulin is an integral subunit of the enzyme, and the calmodulin-binding site may differ from those found in other proteins, which only interact with calmodulin in the presence of Ca^{2+}. A valuable method for identifying the calmodulin-binding site and other functional domains of the γ-subunit would be to employ site-directed mutagenesis of the cDNA coding for this protein and gene expression studies. In order to obtain mutated forms of the γ-subunit cDNA we have isolated and sequenced a cDNA spanning the entire coding region of the phosphorylase kinase γ-subunit.

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The nucleotide sequence presented has been submitted to the EMBL/GenBank database under the accession number Y00684

2. MATERIALS AND METHODS

2.1. *cDNA library construction*

Skeletal muscle from 11-day-old rabbits was frozen in liquid nitrogen. Total RNA was isolated

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by homogenising the frozen tissue in 10 vols/g of 44% phenol/0.5% SDS/25 mM EDTA/75 mM NaCl (pH 8.2) at 0°C. The aqueous phase was re-extracted with buffered phenol (Bethesda Research Laboratories, USA), made 0.25 M in NaCl and the RNA precipitated with 2 vols of ethanol at −20°C. It was redissolved in 20 mM sodium acetate (pH 5) and reprecipitated at 4°C by addition of an equal volume of 4 M lithium chloride. After redissolving in 0.1 M sodium acetate (pH 7) it was precipitated a second time with ethanol. All aqueous solutions used were treated with 0.1% diethylpyrocarbonate and autoclaved. Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography [8] and used as a template for cDNA synthesis under the conditions described by Gubler and Hoffman [9] with the following modifications. First strand synthesis was carried out with 100 μg/ml poly(A)$^+$ RNA, 1600 units/ml avian myeloblastosis virus reverse transcriptase, and the addition of 300 units/ml placental ribonuclease inhibitor (Amersham International, England), 100 μg/ml actinomycin D (Sigma, USA) and 50 mM KCl with 28 mM 2-mercaptoethanol in place of dithiothreitol. On completion of the synthesis of the second strand, the double-stranded cDNA was *Eco*RI methylated and the ends filled in with the Klenow fragment of DNA polymerase 1 [10]. *Eco*RI linkers were ligated to the cDNA [10], and after restriction with *Eco*RI the material was size fractionated on Sepharose CL-4B [11], fractions containing cDNA greater than 500 nucleotides in length being pooled. A cDNA library was constructed by ligating the size selected, double-stranded cDNA into the *Eco*RI site of λgt10. After in vitro packaging, the library was established by transfection of *Escherichia coli* C600 Hfl [10].

2.2. *Oligonucleotide probes and screening of the cDNA library*

Synthetic oligodeoxynucleotide probe mixtures were designed to be complementary to regions of the γ-subunit of phosphorylase kinase showing little or no homology to other known kinases (table 1). The mixtures, which included all possible coding options were synthesized by Celltech (Slough, England). The 5′-ends of the oligonucleotides were labelled using T_4 polynucleotide kinase [11] and the probes used without any further purification.

Screening of the unamplified library was performed in duplicate with both probes using a modification of the Benton and Davis [12,13] plaque hybridization procedure. Hybridizations were performed in 0.9 M NaCl/90 mM Tris-HCl (pH 7.4)/6 mM EDTA/0.5% Nonidet P-40/2 × Denhardt's solution/0.2% SDS/denatured *E. coli* DNA (100 μg/ml)/*E. coli* tRNA (70 μg/ml)/^{32}P-labelled oligonucleotide (0.8 pmol/ml) at room temperature with oligo γ_{252} (table 1) or at 30°C with oligo γ_{199} (table 1). 1 × Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% BSA (Pentax fraction V). Putative positives were plaque purified and their DNA was isolated from plate lysates by formamide extraction following caesium chloride density gradient purification of the phage [14].

2.3. *Subcloning and sequencing of the cDNA insert*

Phage DNA was digested with *Eco*RI, extracted with phenol and used without further purification for ligation of the insert into the *Eco*RI site of Bluescribe pKS-M13$^+$ [15]. Competent *E. coli* JM109 cells were transformed and recombinant plasmid DNA was purified by caesium chloride centrifugation after alkaline lysis of the cells [11]. The cDNA insert was mapped with restriction enzymes and a set of 'nested' deletions was produced using exonuclease III and mung bean nuclease [15]. DNA from the plasmids containing the deletions was prepared by a rapid small scale boiling method [16]. DNA sequencing was performed directly on this double-stranded, supercoiled plasmid DNA [17] using the dideoxy chain termination procedure [18], [α-^{35}S]dATPαS and buffer gradient gels [19]. Ambiguous regions and gaps were clarified by removing restriction fragments and sequencing the religated, truncated recombinant, or by using synthetic oligonucleotides as sequencing primers. Both the 5′- and 3′-ends of the cDNA insert were also checked from large scale, caesium chloride gradient purified DNA.

2.4. *Northern blot analysis*

Poly(A)$^+$ RNA was electrophoresed on agarose gels after denaturation with either glyoxal/dimethylsulfoxide or formaldehyde/formamide

Table 1

Protein sequences chosen and derived oligonucleotides used to screen the rabbit skeletal muscle cDNA library

Ph.K.γ	199	M	N	D	N	H	P	204
mRNA	5′	AUG	AAU C	GAU C	AAU C	CAU C	CCN	3′
Oligo γ 199	3′	TAC	TTA G	CTA G	TTA G	GTA G	GG	5′
Ph.K.γ	252	E	W	D	D	Y		256
mRNA	5′	GAA G	UGG	GAU C	GAU C	UAU C		3′
Oligo γ 252	3′	CTT C	ACC	CTA G	CTA G	AT		5′

[11]. Northern blotting was performed onto Hybond N (Amersham), and blots were prehybridized 2 h at 42°C in 50% deionized formamide/5 × SSC/5 × Denhardt's solution/0.5% SDS/10 mM EDTA/100 μg/ml denatured herring sperm DNA. 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate (pH 7). Hybridizations were performed for 18 h under the same conditions after addition of 1.5×10^6 cpm/ml of 'nick-translated' [11], recombinant plasmid DNA. 15 min washes were performed in 2 × SSC/0.1% SDS; the first being at 42°C and the second at 60°C, with a final stringent wash in 0.2 × SSC/0.1% SDS at 60°C. Hybridizing species were visualized by autoradiography.

3. RESULTS AND DISCUSSION

3.1. *Cloning of the catalytic subunit of phosphorylase kinase*

Screening of 5×10^5 recombinants from an unamplified rabbit skeletal muscle cDNA library prepared in λgt10 yielded only one clone positive with two oligonucleotides complementary to different regions of the γ-subunit coding sequence. The authenticity of the signals was further checked by Southern blot analysis of the *Eco*RI-digested DNA and hybridization to the same oligonucleotides.

3.2. *Sequence analysis*

DNA sequencing confirmed that the clone contained an insert encoding the full length of the γ-subunit. A restriction map and some of the 'nested' deletion clones used for sequencing are shown in fig.1. The 5′-non-coding region, which varies between 20 and 80 nucleotides for the majority of eukaryotic mRNAs, in this instance contained 80 nucleotides. An initiator ATG codon, preceded three nucleotides upstream by an A, conforms with the consensus eukaryotic protein synthesis initiation sequence at these sites [20]. The full coding sequence of 1158 nucleotides is terminated by a TGA stop codon and 614 nucleotides of 3′-untranslated region (fig.2). However the consensus polyadenylation sequence (AATAAA) is missing from the 3′-non-coding region, as well as a poly(A) tail, presumably as the result of a cloning artefact.

The deduced amino acid sequence is in complete agreement with the amino acid sequence determined by Reimann et al. [4], but differs from the N-terminal sequence reported by Crabb and Heilmeyer [21] in that position 21 is proline and not serine. The initiator methionine is absent in the isolated γ-subunit [4,21] and is either removed on formation of the mature γ-subunit or by proteolysis during the purification of the enzyme.

3.3. *Northern blot analysis*

Poly(A)$^+$ RNA, isolated from rabbit skeletal muscle, was shown to contain a major message for the γ-subunit, 2.1 kb in length (fig.3). The discrepancy between the size of the cDNA

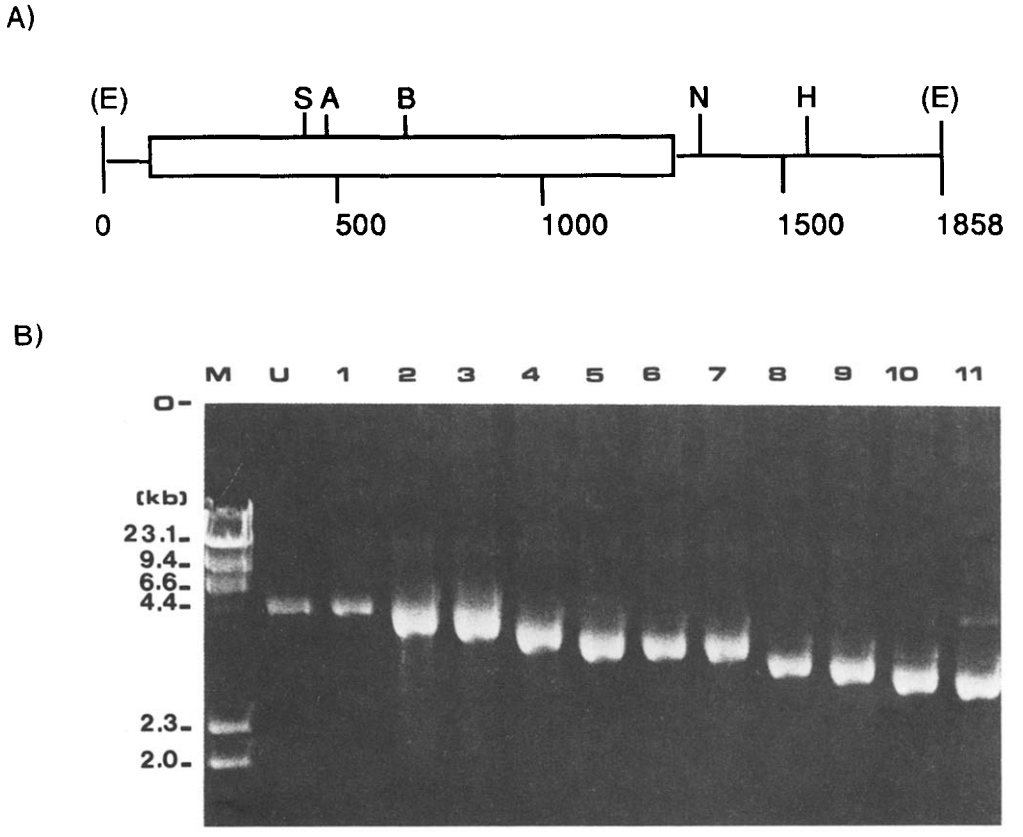

Fig.1. (A) Restriction map of the cDNA for the catalytic subunit of phosphorylase kinase. The letters S, A, B, N and H mark the sites for cleavage by the restriction enzymes *Sac*I, *Apa*I, *Bst*XI, *Nar*I and *Hin*dIII, respectively. (B) Agarose gel showing some of the 'nested' deletions of the cDNA for the catalytic subunit of phosphorylase kinase. Lane M is λ-*Hin*dIII size markers, lane U shows the original Bluescript plasmid containing the cDNA insert for the catalytic subunit of phosphorylase kinase before deletion, and lanes 1–11 show different lengths of deletion.

(1.85 kb) and the size of the mRNA may be accounted for by the missing poly(A) tail.

3.4. *Sequence homology to other protein kinases*

While this work was in progress Hanks [22] reported the isolation of a short cDNA clone (435 nucleotides) from HeLa cells sharing 72% amino acid sequence identity to part of the rabbit skeletal muscle γ-subunit. Comparison of nucleotide sequences shows 71% homology between the HeLa and skeletal muscle clones, indicating a strong evolutionary relationship between the two nucleotide sequences (fig.4). It is possible, therefore, that the HeLa clone codes for the liver type isozyme. Alignment with the cDNA sequence of the catalytic subunit of mouse cAMP-dependent protein kinase [23] gave a value of 45% homology (not shown), over a region spanning amino acids 21–276 of the phosphorylase kinase catalytic subunit sequence, indicating a more distant relationship.

3.5. *Codon usage*

The cDNA coding for the rabbit skeletal muscle γ-subunit exhibits a high G+C content (58%), which is evident in the coding region (57%), as well as in the 5′- and 3′-non-coding regions. Perusal of the codon usage (table 2) reveals that the G+C richness in the coding region is largely explained by the presence of G or C at the third position of each coding triplet (81% are G or C). A similar result was observed for the cDNA of skeletal muscle

```
        GGGGCTCTGGGACCTTTGGGATTCTCGTCAAGCTCATTCCGGGACCTGCAAGGACCTAACGAGCCCCCGAGGAGCTGAGC ATG   83
                                                                                          M

ACCCGCGACGCGGCACTGCCTGGCTCTCAC TCCACACATGGCTTCTACGAGAATTATGAG CCCAAGGAGATCCTGGGCAGAGGAGTTAGC  173
 T  R  D  A  A  L  P  G  S  H   S  T  H  G  F  Y  E  N  Y  E   P  K  E  I  L  G  R  G  V  S

AGTGTGGTCAGGCGCTGCATCCACAAGCCC ACGTGCAAGGAGTATGCCGTGAAGATCATT GATGTCACTGGTGGAGGCAGCTTCAGCGCT  263
 S  V  V  R  R  C  I  H  K  P   T  C  K  E  Y  A  V  K  I  I   D  V  T  G  G  G  S  F  S  A

GAGGAGGTGCAGGAGCTGCGAGAAGCCACG CTGAAGGAGGTGGACATCCTACGCAAGGTC TCGGGGCACCCCAACATCATACAGCTGAAG  353
 E  E  V  Q  E  L  R  E  A  T   L  K  E  V  D  I  L  R  K  V   S  G  H  P  N  I  I  Q  L  K

GACACTTATGAGACCAACACTTTCTTCTTC TTGGTGTTTGACCTGATGAAGAAAGGGGAG CTCTTTGACTACCTCACTGAGAAGGTCACC  443
 D  T  Y  E  T  N  T  F  F  F   L  V  F  D  L  M  K  K  G  E   L  F  D  Y  L  T  E  K  V  T

TTGAGTGAGAAGGAAACCCGAAAAATCATG AGGGCCCTGCTGGAGGTGATCTGCGCTTTG CACAAACTCAACATTGTGCATCGGGACCTG  533
 L  S  E  K  E  T  R  K  I  M   R  A  L  L  E  V  I  C  A  L   H  K  L  N  I  V  H  R  D  L

AAGCCCGAGAACATCCTCTTGGATGATGAC ATGAACATCAAGCTCACAGACTTCGGCTTT TCCTGCCAGCTGGACCCCGGAGAGAAGCTG  623
 K  P  E  N  I  L  L  D  D  D   M  N  I  K  L  T  D  F  G  F   S  C  Q  L  D  P  G  E  K  L

CGAGAGGTCTGTGGGACCCCCAGTTACTTG GCTCCTGAGATCATCGAGTGCTCCATGAAT GACAACCACCCAGGCTATGGGAAGGAGGTG  713
 R  E  V  C  G  T  P  S  Y  L   A  P  E  I  I  E  C  S  M  N   D  N  H  P  G  Y  G  K  E  V

GACATGTGGAGCACAGGGGTCATCATGTAC ACCCTGCTGGCCGGCTCCCCGCCCTTCTGG CACCGGAAGCAGATGCTGATGCTGAGGATG  803
 D  M  W  S  T  G  V  I  M  Y   T  L  L  A  G  S  P  P  F  W   H  R  K  Q  M  L  M  L  R  M

ATCATGAGTGGCAACTACCAGTTTGGCTCA CCGGAATGGGATGATTACTCCGACACGGTC AAGGACTTGGTTTCTCGCTTCTTGGTGGTC  893
 I  M  S  G  N  Y  Q  F  G  S   P  E  W  D  D  Y  S  D  T  V   K  D  L  V  S  R  F  L  V  V

CAGCCCCAGAAGCGCTACACGGCTGAAGAG GCCCTGGCACACCCCTTCTTCCAGCAGTAC GTGGTGGAGGAAGTGCGCCACTTCAGCCCC  983
 Q  P  Q  K  R  Y  T  A  E  E   A  L  A  H  P  F  F  Q  Q  Y   V  V  E  E  V  R  H  F  S  P

CGGGGGAAGTTCAAGGTGATCTGCCTAACC GTGCTGGCTTCGGTGCGCATCTACTACCAG TACCGCCGGGTGAAGCCCGTCACCAGGGAG 1073
 R  G  K  F  K  V  I  C  L  T   V  L  A  S  V  R  I  Y  Y  Q   Y  R  R  V  K  P  V  T  R  E

ATCGTCATCCGGGACCCCTACGCCCTCCGA CCTCTGCGCCGCCTCATCGACGCCTACGCT TTCCGAATCTACGGCCACTGGGTGAAGAAG 1163
 I  V  I  R  D  P  Y  A  L  R   P  L  R  R  L  I  D  A  Y  A   F  R  I  Y  G  H  W  V  K  K

GGGCAGCAGCAGAACCGGGCTGCCCTCTTT GAGAACACCCCCAAGGCTGTGCTCTTCTCG CTGGCTGAGGATGACTACTGA GGGGGCAG 1252
 G  Q  Q  Q  N  R  A  A  L  F   E  N  T  P  K  A  V  L  F  S   L  A  E  D  D  Y  :

GCACGCGGGGGAGGCATGAGGCCCCCGCCAGGAGCCCCCTCCCCGGGTGCTGAAGCCAGCGATGGACGGACCCCGACCCCGGGCCAGGCGCC 1344

ATTCCTGAGGAGGGAGGACCAGATGCGGTCCGGGGCAGAGCCCTTGCTTCGTGGGAGGCGCAAAATCTCTAACACCCCTGCTGCTGTCTTCC 1436

CTGATTCCCAGGGCGAGGCTAAAACACGGGACATGTTTAGAGATGCGCAGTCACACGATGTGCACTGGCATCCGGGGACTTGGTCCGAGAGG 1528

CCCAGGGAAGCCCTTAGGTATATTAGCAATAGAAGAGGAAGCTTCTGATACTTCCTCTGGAGCGTTATTTACTTTGTGACTCCCACGGTGCA 1620

CTGCTTCTCCTGGTCCTTTGGGTCTCCCAGGTCCCCCGTGAAGACTGACGCTGTCCTGTCAAGAGCCACCGTGGTGCATTTTACACGTGGAG 1712

AAAAATCACGGTTCTTCTCAGGGGCATGAAACGCCACAGAGGAGGCCAATGAAATTGCATCTTCCTCCTACAGCGGTTTTCTGGGAAAACAA 1804

AGAAATGGGGACACCCTGCCTTGAGAAAACACTTGTCAGATCCCTAAATCAGAC                                    1858
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Fig.2. The cDNA and translated protein sequence for the catalytic subunit of phosphorylase kinase.

phosphorylase, where the G + C content of the 3rd codon position was found to be 86% [24]. This compares with G + C values of 60% for the 3rd codon position in the cDNA of liver phosphorylase [25] and 70% for the partial cDNA sequence for the putative liver phosphorylase kinase γ-subunit (fig.4). Comparative values for the cDNAs of rabbit skeletal muscle and human liver aldolases are 79 and 58%, respectively [26,27]. The reason for the tissue specific differences in G + C content of the mRNA is not known, although it has been speculated that enhanced G + C content aids

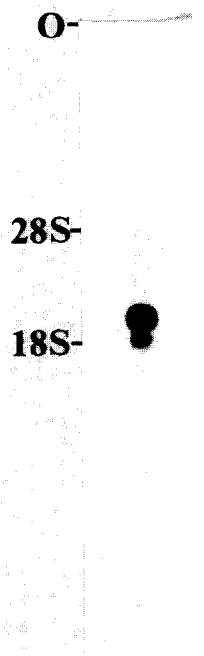

Fig.3. Northern blot of 5 μg of rabbit skeletal muscle poly(A)$^+$ RNA. The probe used was the full length 'nick-translated' cDNA clone in Bluescript. The positions of 28 S and 18 S ribosomal RNA are indicated; O marks the origin.

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Muscle   GAAACCCGAAAAATCATGAGGGCCCTGCTGGAGGTGATCTGCGCT   501
HeLa     ......A.GTCC......C..T.T..C.......CAG.GA..TT.    45

Muscle   TTGCACAAACTCAACATTGTGCATCGGGACCTGAAGCCCGAGAAC   546
HeLa     C.C..TCCCAA..............A..T..A..........T     90

Muscle   ATCCTCTTGGATGATGACATGAACATCAAGCTCACAGACTTCGGC   591
HeLa     ..T...C.A.....CA.T...C.G...CGA..TT....T.....G  135

Muscle   TTTTCCTGCCAGCTGGACCCCGGAGAGAAGCTGCGAGAGGTCTGT  636
HeLa     ..C....C...CT.......T..C........T......T.G...  180

Muscle   GGGACCCCCAGTTACTTGGCTCCTGAGATCATCGAGTGCTCCATG  681
HeLa     ........AG....TC.A..G..A......C.TA.A.........  225

Muscle   AATGACAACCACCCAGGCTATGGGAAGGAGGTGGACATGTGGAGC  726
HeLa     G....A.C..............CTT..T..AC...C.C...GC.   270

Muscle   ACAGGGGTCATCATGTACACCCTGCTGGCCGGCTCCCCGCCCTTC  771
HeLa     TGT....AG...T...T...A..C.....T.....G..A......  315

Muscle   TGGCACCGGAAGCAGATGCTGATGCTGAGGATGATCATGAGTGGC  816
HeLa     .........CG......C......T.AC.C.........GAG...  360

Muscle   AACTACCAGTTTGGCTCACCGGAATGGGATGATTACTCCGACACG  861
HeLa     C.G........CA.T..C..C..G........CCGT...AG...T  405

Muscle   GTCAAGGACTTGGTTTCCCGCTTCTTGGTG                 891
HeLa     .....A...C..A.C..CA.GC.GC..CA.                 435
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Fig.4. Sequence comparison of the cDNAs for the catalytic subunit of phosphorylase kinase from rabbit skeletal muscle and human HeLa cells. The latter are presumed to express the liver type phosphorylase kinase.

stability, which may be important in gene function in muscle, which undergoes greater extremes of temperature and pH than liver [25].

3.6. *Use of the cDNA clone in understanding the molecular basis of phosphorylase kinase deficiencies*

Deficiency of phosphorylase kinase in murine skeletal muscle shows X-linkage [28] and results in loss of activity. The α-, β- and γ-subunits are absent and the amount of calmodulin is reduced [29]. The underlying gene defect may therefore reside in one of the structural genes for the subunits or in a separate X-linked gene that controls their synthesis. However, the gene for the β-subunit has been shown to be located on an autosome in rabbits [30] and the genes for calmodulin have been determined to be autosomal in man (Scambler, P., personal communication). Since polypeptides that are X-linked in one mammal are almost invariably X-linked in others [31], the murine skeletal muscle deficiency is unlikely to be due to a defect in the gene for the β-subunit or calmodulin. Two isozymes of phosphorylase kinase are present in muscle, that possess the structures $(\alpha\beta\gamma\delta)_4$ and $(\alpha'\beta\gamma\delta)_4$ [29]. The α'-subunit is 5 kDa smaller than α, and if it is the product of a separate gene,

Table 2

Codon usage table of the catalytic subunit of rabbit skeletal muscle phosphorylase kinase

F	TTT	5	S	TCT	2	Y	TAT	4	C	TGT	1
F	TTC	14	S	TCC	5	Y	TAC	15	C	TGC	6
L	TTA	0	S	TCA	1	*	TAA	0	*	TGA	1
L	TTG	7	S	TCG	3	*	TAG	0	W	TGG	4
L	CTT	0	P	CCT	3	H	CAT	2	R	CGT	0
L	CTC	9	P	CCC	13	H	CAC	9	R	CGC	10
L	CTA	2	P	CCA	1	Q	CAA	0	R	CGA	5
L	CTG	19	P	CCG	2	Q	CAG	13	R	CGG	6
I	ATT	2	T	ACT	4	N	AAT	2	S	AGT	4
I	ATC	19	T	ACC	9	N	AAC	9	S	AGC	5
I	ATA	1	T	ACA	3	K	AAA	3	R	AGA	1
M	ATG	11	T	ACG	4	K	AAG	23	R	AGG	4
V	GTT	2	A	GCT	9	D	GAT	6	G	GGT	1
V	GTC	10	A	GCC	8	D	GAC	16	G	GGC	10
V	GTA	0	A	GCA	2	E	GAA	5	G	GGA	3
V	GTG	18	A	GCG	1	E	GAG	24	G	GGG	7

then the defect cannot be due to a point mutation in both the α and α' genes, since the deficiency shows a single gene mode of inheritance. However other interpretations are possible; for instance, the mRNAs for the α and α'-subunits may arise by differential splicing of a common message. The availability of a cDNA probe for the γ-subunit will allow us to test whether the gene for the γ-subunit is located on the X-chromosomes. The probe may also be useful in the investigation of human phosphorylase kinase deficiencies. The majority of these also show X-linkage and affect the liver, while a few show autosomal inheritance and affect both liver and muscle [32].

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council, London, and the Muscular Dystrophy Group in Great Britain.

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