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Complete nucleotide sequence of dog heart creatine kinase mRNA: Conservation of amino acid sequence within and among species

(sequence homology/gene family/myocardial infarction/protein compartmentation)

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ABSTRACT **Creatine kinase (CK; EC 2.7.3.2) plays an important role in energy metabolism in brain and muscle. Expression of CK isoenzymes is regulated during development and is tissue specific. To define the structures of canine CK isoenzymes and to elucidate the mechanism of regulation in their expression, CK cDNA clones from dog myocardium were isolated. Myocardial CK mRNA is predicted to encode a protein of 381 amino acids. The nontranslated regions of the mRNA comprise at least 38 bases at the 5′ end and exactly 345 bases before the poly(A) tail. Partial protein sequences of dog muscle (M) CK and brain (B) CK subunits were determined and compared with the derived amino acid sequence of the myocardial enzyme and of M CK subunits of other species. The M CK subunits from different species share a very high degree (83–96%) of sequence identity. Dog M and B subunits share extensive sequence identity (74%), a degree of similarity not previously suspected. Southern blot analysis suggests that a CK gene family exists. These observations imply that evolutionary changes in the M CK subunit structure are constrained by the need for preservation of functional properties other than the kinase activity. This conservation is consistent with the possibility that the M subunit plays a structural role in cardiac and skeletal muscle.**

Creatine kinase (CK) (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) catalyzes the reversible transfer of a phosphate residue between phosphocreatine and ADP and plays a prominent role in cardiac and skeletal muscle energy metabolism (1). Assay of plasma CK isoenzyme activity and of isoforms derived from individual isoenzymes has proven useful for diagnosis and characterization of the course and extent of myocardial infarction (2, 3). Determination of the structural basis of plasma CK heterogeneity is of potential clinical, as well as biochemical, interest.

Several aspects of the intracellular compartmentation, biosynthesis, and developmental regulation of CK make it an intriguing focus for study in terms of both genetic organization and protein structure. Both cytosolic and mitochondrial forms of the enzyme are synthesized in the cytosol from mRNAs encoded by the nuclear genome (4). The mitochondrial form is incorporated into the mitochondrial inner membrane after post-translational proteolytic processing from a larger precursor. The enzyme present in the cytosol is a dimer consisting of subunits designated as M or B because of the relative preponderance of each in muscle and brain, respectively. The MM isoenzyme predominates in skeletal muscle. The BB homodimer is the major species in brain. The MB heterodimer constitutes a variable portion of the total CK activity found in smooth and cardiac muscle of some species (1). In skeletal muscle, approximately 5% of the MM CK is associated with the myofibrillar apparatus at the M band of the sarcomere (5). This localized CK is believed to act both as an intramyofibrillar ATP-regenerating system and as a structural component of the M bridges, because it is their principal protein constituent (5, 6). Thus, CK gene products are present in two compartments and localized to two discrete domains in the cytosol.

Until recently, little information concerning the primary structure of the isoenzymes of CK was available. However, results of peptide mapping, analyses of amino acid composition, and assay of immunologic reactivity have shown that MM isoenzymes from different species are structurally more similar than the M and B subunits from the same species (7). Although the M CK subunit is highly conserved with regard to size and proteolytic cleavage sites, the B subunits exhibit considerable heterogeneity of size among species (7). MM and BB CKs exhibit similar enzyme kinetics (1, 5), and dissociation of mixtures of M and B subunits under denaturing conditions followed by renaturation indicates that heterodimers with normal enzymatic function can be formed (7). Thus, despite apparent differences in primary structure, both subunits exhibit considerable structural similarity.

Expression of CK isoenzymes is developmentally and hormonally regulated within various tissues (5, 8). For example, 17β-estradiol induces the synthesis of BB CK in rat myometrium (9). For skeletal muscle undergoing maturation, MM CK content increases. Concomitantly, the BB isoenzyme declines to undetectable levels in adult muscle (5). In cardiac muscle, expression of B CK may be reduced as activation of the M gene occurs. This switchover varies quantitatively among species (5, 10). Although transcriptional control of M CK gene expression has been documented (5), the mechanism of the isoenzyme switch has not been elucidated.

The cDNA-derived amino acid sequences of skeletal muscle CK from several species have recently been reported (11–16). However, no amino acid sequence information is available for the cardiac M subunit. Other myofibrillar proteins, such as actin and myosin, exist in tissue-specific, although highly homologous, forms (17). It has been suggested that multiple forms of skeletal muscle CK exist, as reflected by results of isoelectric focusing and NH_2-terminal sequence analyses (5, 18). Therefore, we have determined the complete amino acid sequence of the M subunit of canine myocardial CK, using cDNA cloning and protein sequence information. Comparison with skeletal muscle M CK subunits from other species and with partial sequences from canine BB CK demonstrates a very high degree of sequence conservation throughout the molecule, suggesting that domains other than the enzymatic active site may be essential for previously unsuspected functions of the protein.

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Abbreviations: CK, creatine kinase; M and B, CK subunits preponderant in muscle and brain, respectively; bp, base pair(s), kb, kilobase(s).

MATERIALS AND METHODS

Isolation of RNA, *in Vitro* Translation, and cDNA Library Preparation. mRNA was prepared from canine myocardium by the proteinase K technique (19). Total cellular RNA and poly(A)-containing RNA were translated *in vitro* in a wheat germ cell-free lysate (4). Radiolabeled M CK was precipitated from the translation reactions with antibody and *Staphylococcus aureus* protein A as previously described (4). Poly(dC) tails were added to double-stranded cDNA (20), which was annealed to tailed and *Pst* I-digested pBR322 and used to transform the RR1 strain of *Escherichia coli* (21). The library was screened (22) with a 450-base-pair (bp) chicken M CK clone (kindly provided by Charles Ordahl) as a probe (11).

Restriction Analyses and Determination of DNA Sequences. Restriction enzyme analysis of plasmid DNA (23) was performed under standard conditions. Nucleic acid sequencing was by the dideoxy chain-termination method (24) after subcloning in M13 phages. Either the universal M13 sequencing primer or synthetic oligonucleotides (25) derived from CK cDNA were used for sequence determinations. DNA extraction from dog spleen, dot blot, Southern blot, and RNA blot analyses were performed according to methods previously described (22).

Determinations of Protein Sequence. MM CK was purified from canine heart (26) to a specific activity of >700 international units per mg of protein. BB CK was purified from dog brain to a specific activity of 650 international units per mg of protein. Both proteins were greater than 95% pure. The B and M subunits were cleaved with cyanogen bromide or treated with trypsin or staphylococcal V8 protease. The resulting peptides (0.5–2 nmol) were separated by HPLC and subjected to automated Edman degradation (27). Phenylthiohydantoin amino acids were identified by HPLC (28).

RESULTS

Isolation of M CK Clones from a cDNA Library Derived from Dog Heart. *In vitro* translation of dog heart RNA in a wheat germ lysate, followed by immunoprecipitation, gel electrophoresis and autofluorography, was utilized to assess the relative abundance of M CK mRNA. A single radiolabeled band was visualized, with mobility identical to that of the purified enzyme, apparent M_r = 43,000 (data not shown). The abundance of the mRNA estimated by the incorporation of label as a percentage of total protein synthesis was 0.1%.

Screening of a cDNA library of 5×10^4 independent recombinants with a 450-bp chicken M CK probe (11) yielded 64 positive signals. Three putative CK clones, pCKR10, pCKR21, and pCKR94, were subjected to restriction analysis and DNA sequence analysis (Fig. 1). The three clones are overlapping and together comprise the entire coding sequence of canine myocardial CK, all of the 3′ nontranslated region (345 bp), and 38 nucleotides of the 5′ nontranslated region (Figs. 1 and 2). Comparison of the shared sequences within the three clones demonstrated complete identity, suggesting that they were derived from the same species of mRNA.

The composite complete nucleotide sequence of canine M CK mRNA with the derived amino acid sequence is displayed in Fig. 2. Prior to the poly(A) tail, 1529 nucleotides of sequence were determined. The initiation codon was identified at position 43 as part of the consensus sequence proposed by Kozak -CC$\frac{A}{G}$CCATG (29). No ATG sequences were found prior to this site. Initiation of translation at nucleotide 43 generates an open reading frame of 381 amino acids of aggregate M_r = 43,138, consistent with the estimated M CK subunit size. The other two reading frames contain numerous earlier stop codons. The stop codon (TAG) has been identified at position 1144 and the polyadenylylation signal sequence AATAAA appears at nucleotide 1468, 14 bp prior to a poly(A) sequence.

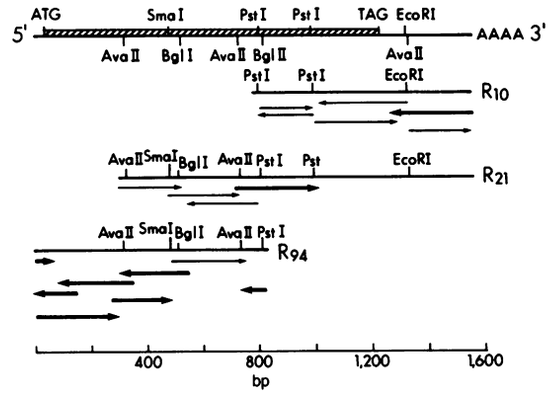

FIG. 1. Partial restriction map and sequencing strategy for clones pCKR10, pCKR21, and pCKR94. The map does not include poly(dG·dC) tails but is otherwise drawn to scale. Thin arrows indicate sequences determined for fragments cloned in mp13 vectors, using the universal primer. Thick arrows indicate sequence data obtained in subclones, using synthetic oligonucleotide sequences of the inserts themselves as primers. Although certain regions of clone R21 were not sequenced, overall both strands were sequenced in their entirety.

Determination of Partial Amino Acid Sequences of Dog M and B CK. To confirm the amino acid sequence derived from the myocardial cDNA clones, intact canine cardiac CK and peptides derived from it after cyanogen bromide and tryptic cleavage were subjected to NH_2-terminal sequence analyses (Fig. 2). Among the 121 residues determined, three assignments were uncertain, probably because of the low yields of histidine and tryptophan on the gas-phase sequencer. All of the remaining residues corresponded exactly to the sequences predicted from the cDNA clones. Treatment of dog MM CK with carboxypeptidase B followed by digestion with cyanogen bromide generated a peptide map identical to that of mature MM CK except for the single peptide with the sequence of residues 376–380 (Fig. 2). This peptide (30) lacks the terminal lysine. Thus, translation of M CK mRNA indeed stops at the predicted site. These data demonstrate that the predicted amino acid sequence is correct and that the cDNA encodes the myocardial M subunit of CK.

A partial amino acid sequence of canine BB CK has also been determined from the intact protein and from tryptic and cyanogen bromide fragments derived from it (Fig. 3). Surprisingly, in neither M nor B CK subunits is the NH_2 terminus blocked (31). Sequences of B CK determined from nine fragments and the NH_2 terminus, comprising 183 residues in aggregate, were compared with the canine M CK sequence. For most of the peptides homology was between 75% and 90%, enabling us to construct alignments with confidence. The homology between M and B subunits was 74%. The partial sequence of BB CK encompassed virtually half the molecule. The data demonstrate that the M and B subunits of canine CK share an unexpectedly high degree of amino acid sequence identity.

While this work was in progress, cDNA clones and derived amino acid sequences for various M CK subunits were reported (11–16). Comparison of the six reported sequences is shown in Fig. 3 and Table 1. Sequence identity is 86–96%. If conservative substitutions are allowed, the extent of similarity approaches 98% for all species. Among the three mammalian sequences, half of the amino acid substitutions occur within the first 45 residues, suggesting that this region is less important for conserving the functional properties of

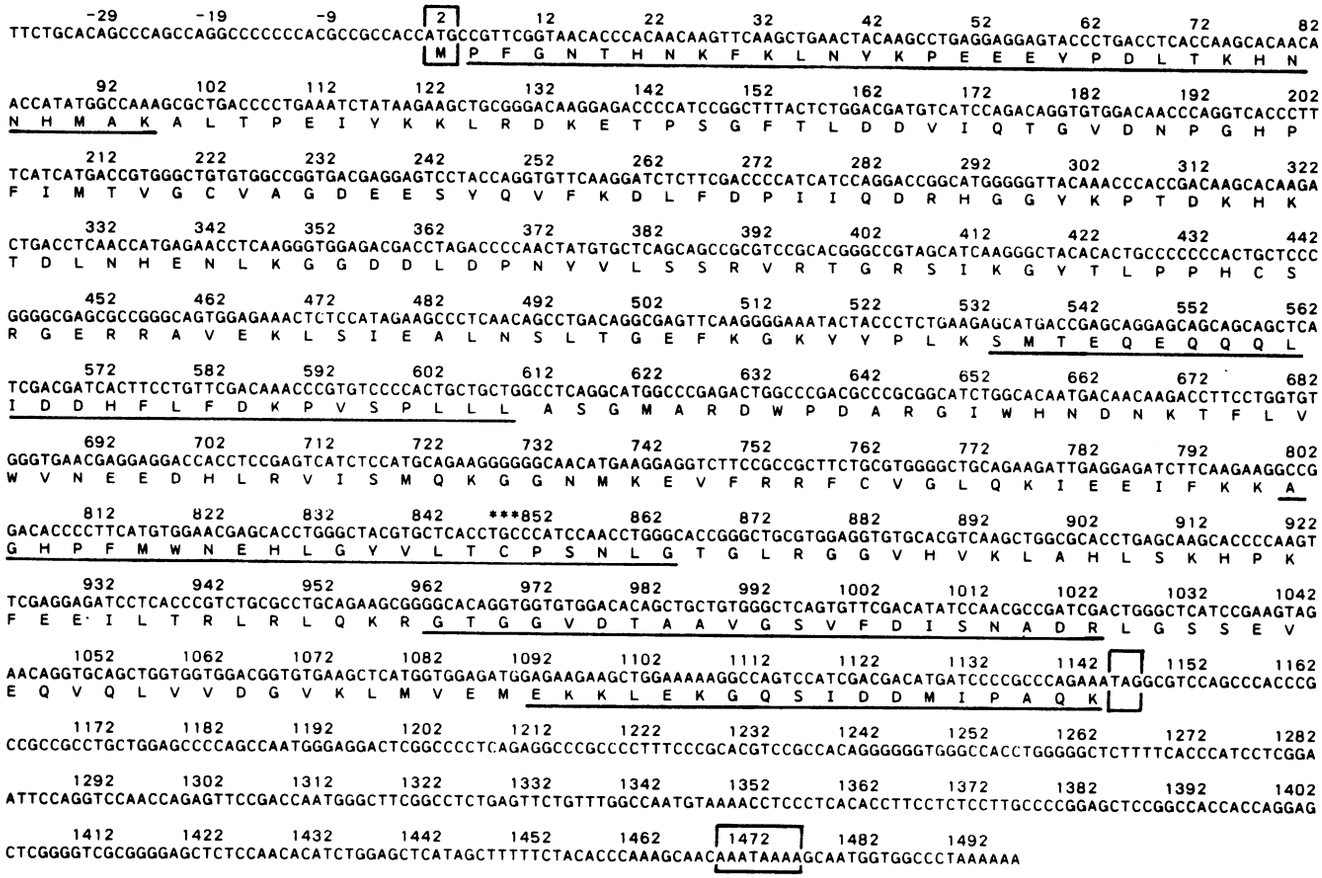

FIG. 2. Complete nucleotide sequence of dog myocardial CK with the derived amino acid sequence. The single-letter code for amino acids is shown. Numbering indicates nucleotides and begins at the initiation codon, with negative numbers to show the 5′ noncoding region. The cysteine near the active site is indicated by an *. The start, stop, and poly(A) addition sites are outlined with boxes. Only 6 of the >30 adenosine residues in the tail are shown. Predicted amino acid sequences confirmed by automated degradation of peptides derived from myocardial CK are underlined.

the enzyme. In contrast, less than 2 substitutions per hundred residues occur over the remaining 335 amino acids. In the mammalian enzymes, all of these later amino acid replacements occur in the same positions (residues 82, 99, 123, 158, 223, 240, 300, and 331) and are highly conservative. Comparison between canine M CK and the nonmammalian species—i.e., *Torpedo* and chicken, reveals a similar pattern. Again, the region of greatest variability is at the NH_2 terminus and the specific residues listed are sites of substitutions.

Chemical modification of a cysteine residue in CK alters enzymatic activity (32). This cysteine corresponds to residue 282 of the predicted CK sequence. The lysine and histidines necessary for phosphate group transfer and the arginine and tryptophan involved in nucleotide binding have not been identified (11). Among the 14 histidine residues in canine CK, 10 are identical in all the species shown (Fig. 3). Similarly, all of the tryptophan residues are conserved. Thus, determination of which residues are required for activity must await

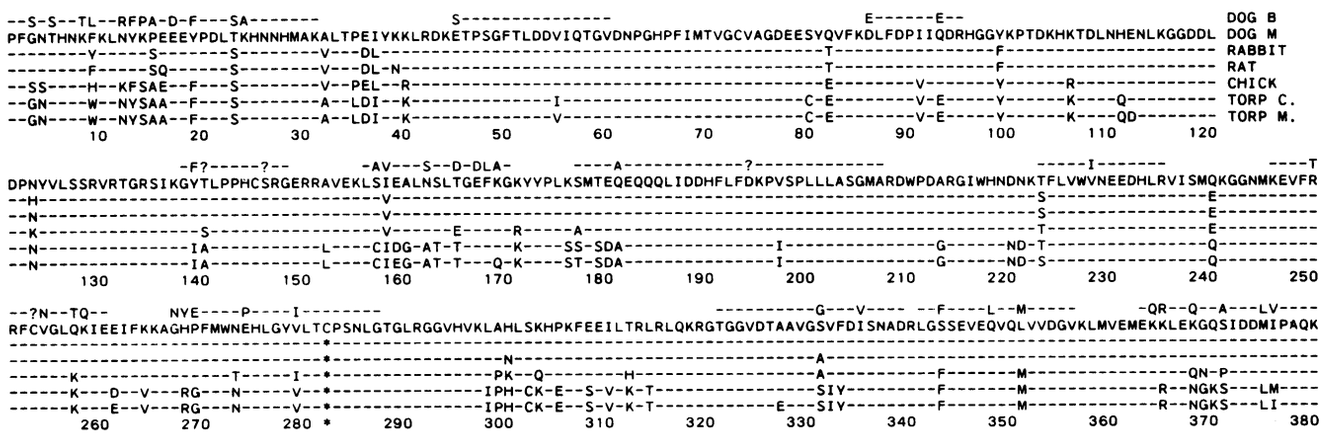

FIG. 3. Alignment of creatine kinases at the amino acid level. Numbering is from the proline at position 2 of the cDNA-derived sequences. A - indicates identity with the canine M sequences; an * indicates the essential cysteine near the active site. Substitutions are shown by the single-letter code for amino acids. The data are from refs. 11–16. Canine B CK amino acid sequences shown above the M subunit sequence were determined by automated Edman degradation of peptides derived from BB CK. Torp C., *Torpedo californica*; Torp M., *Torpedo marmorata*.

Table 1. Sequence homology among CK M subunits within the coding region

	% homology					
	Dog	Rabbit	Rat	Chicken	*T. californica*	*T. marmorata*
Dog		96.8	96.3	90.8	86.1	85.5
Rabbit	91.9		98.4	92.9	85.0	85.5
Rat	90.1	90.9		90.8	84.5	84.7
Chicken	83.1	84.1	82.6		83.4	83.4
T. californica	79.9	79.7	79.5	78.2		97.6
T. marmorata	79.9	79.3	79.6	77.5	98.1	

Sequence data were obtained from refs. 11–16. Numbers above and right of the blank diagonal are amino acid homology; numbers below and left are nucleotide homology.

crystallographic data or creation of site-specific mutations.

Blot Hybridization Analyses. Hybridization analysis of dog heart RNA was performed with an 835-bp fragment corresponding to the COOH-terminal two-thirds of the protein and 100 bp of untranslated region (Fig. 4). Analysis demonstrated a single species of hybridizing mRNA migrating in the region of 1.6 kb, consistent with the predicted size of the M CK mRNA (Fig. 2). Thus, the clones encompass virtually all of the mRNA, although the precise size of the 5′ nontranslated region of the mRNA was not determined. Southern blot analysis of genomic DNA extracted from dog spleen was performed with the same probe after digestion of the DNA with *Eco*RI, *Hin*dIII, or *Bam*HI. Multiple hybridizing bands were detected in all three digests. Identical results were obtained after Southern blot analysis using a 180-bp probe corresponding to amino acids 256–318 (data not shown). These results could reflect (*i*) incomplete digestion of the DNA by the restriction enzymes, (*ii*) the presence of a large number of introns within the dog M CK gene containing *Eco*RI and *Bam*HI restriction enzyme sites, or (*iii*) hybridization of the probe to multiple CK genes. Incomplete digestion is unlikely since a fatty acid binding protein cDNA probe bound to only one fragment of genomic DNA (data not shown). Because no sites for these enzymes are present in the coding region of dog M CK, the second possibility is unlikely unless a large number of long introns were present within the gene. Thus, it appears that the hybridization probe is binding to more than one CK gene. In view of the extensive amino acid homology between M andB CK subunits, such cross-hybridization seems likely.

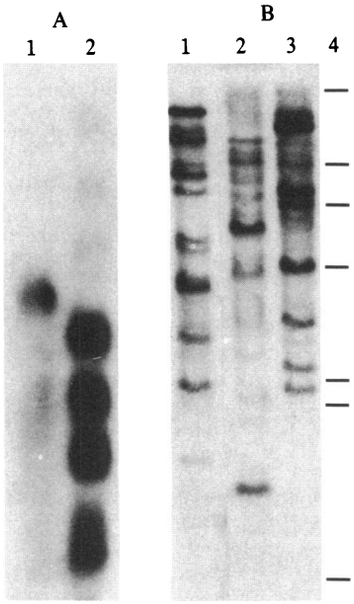

FIG. 4. Blot hybridization analyses with myocardial CK cDNA fragment (*Eco*RI–*Sma* I, 835 bp) and poly(A)-containing RNA from dog heart (*A*) or DNA from dog spleen (*B*). In *A*, 15 μg of heart RNA separated by electrophoresis was transferred to nitrocellulose and probed with the cDNA fragment (lane 1). The migration on the same gel (lane 2) of labeled DNA markers [1.3, 1.0, 0.8, and 0.6 kilobases (kb), respectively, top to bottom] is shown. In *B*, dog spleen DNA was cut with *Eco*RI (lane 1), *Bam*HI (lane 2), or *Hin*dIII (lane 3) prior to electrophoresis, transfer, and hybridization. The migration on the same gel (lane 4) of unlabeled DNA markers (23.1, 9.4, 6.5, 4.3, 2.3, 2.0, and 0.5 kb, respectively) is indicated.

DISCUSSION

We have determined (Fig. 2) the complete CK cDNA sequence derived from the mRNA of cardiac muscle. In view of the immunologic cross-reactivity between canine heart and skeletal muscle CKs, it is likely that myocardial CK is identical or very similar to canine skeletal muscle M CK. The homology of the derived dog heart CK sequence with other mammalian skeletal muscle CKs (Fig. 3) supports this conclusion. However, until heart and skeletal muscle CK from the same species are compared, tissue-specific differences among M CKs cannot be excluded completely.

We undertook cloning of myocardial CK, rather than skeletal muscle M CK, in part because we wished to determine the structural differences among isoforms of MM and MB CK found in plasma after release of CK from canine and human myocardium undergoing infarction (3, 30). Comparison of peptide maps from cardiac CK isoforms with the sequence predicted from the cDNA clone reveals that the single peptide that differs among the isoforms is the COOH-terminal peptide and that this difference reflects the loss of the terminal lysine residue (30). Differences in primary sequence at the COOH terminus of the M and B subunits should permit design of immunologic reagents specific for each isoenzyme and for each isoform useful for the early detection of myocardial infarction.

Another objective of this study was to compare the primary sequences of M and B CK. The availability (11–16) of complete amino acid sequences of M CK from several disparate species permits a detailed comparison of primary structures (Fig. 3). The very high degree of sequence identity across a wide span of evolution suggests that much of the CK molecule is required for one or more functions. The kinase activity alone is unlikely to constrain evolutionary changes to such an extent. Perhaps, in addition to its enzymatic function, CK is a structural protein, much like other highly conserved muscle proteins such as actin and myosin. This speculation is supported by comparison of the differences in nucleotide sequences among species, by the finding of compartmentation of CK within the sarcoplasm, and by comparison of the CK isoenzyme sequences.

Comparison of the nucleotide sequences of the M subunit mRNAs (Fig. 3 and Table 1) shows that the 3′ noncoding regions have no significant nucleotide sequence homology and vary considerably in length, from 345 bases for the canine mRNA to 113 bases for the chicken mRNA. Comparison of the 5′ nontranslated regions is less definitive but it reveals obvious homology among the mammalian species that is lacking for the chicken and *Torpedo* cDNAs. The lack of homology in the noncoding regions is expected in the absence of selective pressure during evolution. This contrasts to the very high (78–98%) nucleotide conservation observed throughout the coding region (Table 1), consistent with severe constraint upon alteration in amino acid sequence.

Additional evidence supporting a structural role for MM CK is that a portion of cytosolic MM CK is localized to M bands in the sarcomere, where it is the predominant protein

found in M bridges (5, 6). Sequences within the M subunits, other than those required for the kinase activity, may be essential determinants of this localization through interactions with other proteins in the M bands. Such a function might account for the highly conserved primary structure.

Comparison of partial amino acid sequences of canine MM and BB CKs (Fig. 3) indicates that the M and B gene products are more divergent than the M subunits from various species. Although definitive comparisons must await completion of the B sequence, it is apparent that canine M and B CK will differ by at least 20% in primary structure. Because the M and B subunits exhibit similar enzyme kinetics, this degree of difference in amino acid sequence, compared with that among M subunits from different species, supports the concept that the MM homodimer may have other important functions in muscle cells. As with the M subunits, numerous substitutions between M and B occur near the NH_2 terminus, but differences in amino acids are present throughout the two subunit proteins. Thus, M and B subunit mRNAs must be derived from different genes, rather than by differential splicing of a precursor mRNA derived from the same region of DNA. Although M and B CKs are encoded by different genes, the extent of amino acid identity between the two is quite striking. Moreover, many of the substitutions are conservative. These results are not surprising, especially in view of the facility with which the two subunits form enzymatically active heterodimers. However, the extent of homology has not been suspected previously because of the lack of immunologic cross-reactivity and because of marked differences in peptide maps and variability in B subunit mass (1, 5, 7).

Because the CK subunits share both interspecies and intraspecies homology, it is probable that canine M CK nucleotide probes will hybridize to dog B CK mRNA and to genomic DNA from other species. Although the primary structure of mitochondrial CK has not been defined, cross-hybridization may occur with genomic sequences for this protein as well. The Southern blots of dog genomic DNA support this prediction and are consistent with hybridization to more than one gene. Thus, a CK gene family appears to exist.

The isolation of cDNA clones for M and B CKs should facilitate elucidation of the mechanism of regulation of CK isoenzyme gene expression that occurs during development and definitive determination of the number of genes within the CK gene family.

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1. Watts D. C. (1973) in *The Enzymes*, ed. Boyer, P. D., (Academic, New York), 3rd Ed., Vol. 8, pp. 383–455.
2. Sobel, B. E. & Shell, W. E. (1975) in *Progress in Cardiology*, eds. Yu, P. N. & Goodwin, S. F. (Lea and Feibiger, Philadelphia), Vol. 4, pp. 165–191.
3. Hashimoto, H., Abendschein, D. R., Strauss, A. W. & Sobel, B. E. (1985) *Circulation* **71**, 363–369.
4. Perryman, M. B., Strauss, A. W., Olson, J. & Roberts R. (1983) *Biochem. Biophys. Res. Commun.* **110**, 967–972.
5. Eppenberger, H. M., Perriard, J.-C. & Wallimann, T. (1983) in *Current Topics in Biological and Medical Research*, ed. Rattazzi, M. (Liss, New York), Vol. 7, pp. 19–38.
6. Wallimann, T., Schlosser, T. & Eppenberger, H. M. (1984) *J. Biol. Chem.* **259**, 5238–5246.
7. Perryman, M. B., Strauss, A. W., Buettner, T. L. & Roberts, R. (1983) *Biochim. Biophys. Acta* **747**, 284–290.
8. Eppenberger, H. M., Eppenberger, M., Richterich, R. & Aebi, H. (1964) *Dev. Biol.* **10**, 1–16.
9. Reiss, N. A. & Kaye, A. M. (1981) *J. Biol. Chem.* **256**, 5741–5749.
10. Schweinfest, C. W., Kwiatowski, R. W. & Dottin, R. P. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4997–5000.
11. Ordahl, C. P., Evans, G. L., Cooper, T. A., Kunz, G. & Perriard, J.-C. (1984) *J. Biol. Chem.* **259**, 15224–15227.
12. Kwiatkowski, R. W., Schweinfest, C. W. & Dottin, R. P. (1984) *Nucleic Acids Res.* **12**, 6927–6933.
13. Benfield, P. A., Zivin, R. A., Miller, L. S., Sowder, R., Smythers, G. W. Henderson, L., Oroszlan, S. & Pearson, M. L. (1984) *J. Biol. Chem.* **259**, 14979–14984.
14. Putney, S., Herlihy, W., Royal, N., Pang, H., Aposhian, H. V., Pickering, L., Belagaje, R., Biemann, K., Page, D., Kuby, S. & Schimmel, P. (1984) *J. Biol. Chem.* **259**, 14317–14320.
15. West, B. L., Babbit, P. C., Mendez, B. & Baxter, J. D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7007–7011.
16. Giraudat, J., Devillers-Thierry, A., Perriard, J.-C. & Changeux, J.-P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7313–7317.
17. Schubert, D. (1984) *Developmental Biology of Cultured Nerve, Muscle, and Glia* (Wiley, New York), pp. 26–71.
18. Takasawa, T., Onodera, M. & Shiokawa, H. (1983) *J. Biochem.* **93**, 389–395.
19. Dillmann, W. H., Barrieux, A., Neeley, W. E. & Contreras, P. (1983) *J. Biol. Chem.* **258**, 7738–7745.
20. Gubler, U. & Hoffman, B. J. (1983) *Gene* **25**, 263–271.
21. Hanahan, D. (1983) *J. Mol. Biol.* **166**, 557–580.
22. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
23. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1515–1523.
24. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
25. Caruthers, M. H. & Matteucci, M. D. (1981) *J. Am. Chem. Soc.* **103**, 3183–3186.
26. Grace, A. & Roberts, R. (1982) *Clin. Chim. Acta* **123**, 59–71.
27. Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990–7999.
28. Gordon, J. I., Smith, D. P., Andy, R., Alpers, D. H., Schonfeld, G. & Strauss, A. W. (1982) *J. Biol. Chem.* **257**, 971–978.
29. Kozak, M. (1984) *Nucleic Acids Res.* **12**, 857–871.
30. Billadello, J. J., Roman, D. G., Grace, A., Sobel, B. E. & Strauss, A. W. (1985) *Clin. Res.* **33**, 170 (abstr.).
31. Lebherz, H. G., Bates, O. J. & Bradshaw, R. A. (1984) *J. Biol. Chem.* **259**, 1132–1135.
32. Thompson, A. R., Eveleight, J. W. & Miles, B. J. (1964) *Nature (London)* **203**, 267–269.