Isolation and Characterization of the Gene and cDNA Encoding Human Mitochondrial Creatine Kinase*

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Robert C. Haas†, Clifford Korenfeld, Zhifang Zhang, Benjamin Perryman‡, Dragos Roman, and Arnold W. Strauss¶

From the Departments of Biological Chemistry, Medicine, and Pediatrics, Washington University School of Medicine, St. Louis, Missouri 63110 and the §Department of Medicine, Baylor University School of Medicine, Houston, Texas 77030

Creatine kinase (CK; EC 2.7.3.2) isoenzymes play prominent roles in energy metabolism. Nuclear genes encode three known CK subunits: cytoplasmic muscle (MCK), cytoplasmic brain (BCK), and mitochondrial (MtCK). We have isolated the gene and cDNA encoding human placental MtCK. By using a dog heart MCK cDNA-derived probe, the 7.0-kb EcoRI fragment from one cross-hybridizing genomic clone was isolated and its complete nucleotide sequence determined. A region of this clone encoded predicted amino acid sequence identical to residues 15–26 of the human heart MtCK NH₂-terminal protein sequence. The human placental MtCK cDNA was isolated by hybridization to a genomic fragment encoding this region. The human placental MtCK gene contains 9 exons encoding 416 amino acids, including a 38-amino acid transit peptide, presumably essential for mitochondrial import. Residues 1–14 of human placental MtCK cDNA-derived NH₂-terminal sequence differ from the human heart MCK protein sequence, suggesting that tissue-specific MtCK mRNAs are derived from multiple MtCK genes. RNA blot analysis demonstrated abundant MtCK mRNA in adult human ventricle and skeletal muscle, low amounts in placenta and small intestine, and a dramatic increase during in vitro differentiation induced by serum-deprivation in the non-fusing mouse smooth muscle cell line, BC3H1. These findings demonstrate coordinate regulation of MtCK and cytosolic CK gene expression and support the phosphocreatine shuttle hypothesis.

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‡Fellow of the American Heart Association, Missouri Affiliate.

To whom correspondence and reprint requests should be addressed.

1The abbreviations used are: CK, creatine kinase; M, muscle; B, brain; Mt, mitochondrial; bp, base pair(s); kb, kilobase pair(s); SDS, sodium dodecyl sulfate.

been described. All have similar molecular weights (Mr = 82,000–86,000) and are dimers (2–4). Based upon the lack of immunological cross-reactivity and differences in amino acid composition, electrophoretic mobility, and peptide maps of purified subunits, it has been proposed that three CK genes exist (5–7). These genes encode the three known protein subunits, designated as M (muscle), B (brain), and mitochondrial (Mt), which form three dimeric cytosolic (MM, BB, and MB) and a distinct mitochondrial (MtMt) isoenzyme.

Several aspects of the intracellular compartmentation, biosynthesis, and regulation of CK subunit synthesis make this gene family an excellent model for study of gene structure and regulation of expression during development and muscle differentiation. For example, MtCK activity is not detected in fetal mouse heart, but is detected in the early post-natal period (8). However, MtCK activity is detected in fetal lamb heart in small amounts and undergoes further induction post-natally (9). The cytosolic CKs also undergo developmental transitions. Total cytosolic CK activity increases as much as 400-fold during the terminal differentiation of muscle cells (10) in which myoblasts fuse into multinucleated myotubes. In addition, differentiating skeletal muscle tissue and myogenic cell cultures exhibit an overlapping isoenzyme transition from BCK in embryonic myoblasts to predominantly MCK in adult tissue and myotubes (11, 12). In the non-fusing muscle cell line, BC3H1, we have demonstrated a greater than 100-fold increase in MCK mRNA by Northern blot analyses during growth arrest and differentiation induced by reduction of serum levels (13). Reinstitution of cell division by fibroblast growth factor in BC3H1 cells shuts off MCK gene expression (13). Declining levels of MCK and MtCK have been reported in hypertrophied rat hearts (14) and BCK induction may occur after ischemia or infarction (15, 16). Thus, CK gene expression is regulated during development, muscle differentiation and in various cardiovascular diseases.

We and others have isolated cDNA clones encoding the M and B subunits of CK (17–22). We now report the characterization of the human placental MtCK cDNA and gene. MtCK accounts for 7–48% of the total cellular CK activity in adult skeletal muscle, heart, and brain (3, 23). One model (1) suggests that MtCK couples to the respiratory chain by conversion of ATP generated by oxidative phosphorylation to phosphocreatine. In muscle, for instance, phosphocreatine diffuses readily to the myofibrillar M-band where 5% of MCK is localized (24). ATP can then be regenerated from phosphocreatine for use in muscle contraction. This high energy phosphate shuttle may be necessary for efficient transport of energy equivalents from mitochondria to cytosol.

To compare the structures of cytosolic and MtCK genes, define the regulation of their expression and study the biosynthesis and processing of the MtCK precursor, we have
isolated the human placental MtCK gene and cDNA and determined their complete sequences.

MATERIALS AND METHODS

CK Protein Purification and NH2-terminal Sequence Determination—Human BCK and MCK were purified from brain and heart, respectively, as described previously (25). MtCK was isolated from human myocardium by a modification of a reported method (7). Purity of all CK isoenzymes as assessed by denaturing gel electrophoresis was greater than 95%. The purified proteins were subjected to NH2-terminal sequence analyses in an Applied Biosystems gas phase sequencer using the "no vac" program supplied by the manufacturer. Identification and quantification of the phenylthiohydantoin derivatives released in each cycle were by high performance liquid chromatography as described previously (26).

Isolation and Mapping of CK Genes—The 500-base pair (bp) Smal-EcoRI fragment from dog heart MCK cDNA pCKR21 (17) was used as a hybridization probe to screen a human genomic library constructed in the EcoRI site of λ Charon 4A. This fragment includes 700 bp of coding region and 100 bp of 3′-nontranslated region. All DNA fragment probes were labeled with 32P by the random primer method (27). Single, double, and triple restriction enzyme digests of each individual genomic clone were made after electrophoresis and transfer to nitrocellulose paper as described previously (28). Restriction maps were generated by probing these Southern blots with 5′-, active site-, and 3′-specific MCK cDNA fragments.

Isolation of the Human Placental MtCK cDNA—A 312-base pair KpnI-BamHI fragment from a human genomic clone XhCK39 was used as a hybridization probe to screen a human placenta-derived cDNA library constructed in λgt11. This fragment encodes 186 bp of MtCK coding region and 114 bp of flanking intron sequence. It has a sequence identity of 60% with homologous regions of both BCK and MCK (29) human genes (50). Specificity of this probe for MtCK was demonstrated by dot blot hybridization (30). The identities of selected cDNA clones were determined directly by sequence analysis.

DNA Sequence Analysis—The sequence of the 1.5-kb EcoRI insert derived from clone pGMtCK was determined by the deoxyribonucleotide chain termination method (31) after subcloning into the pGEM3zf+ plasmid. The complete sequence of six contiguous segments of a 7.0-kb EcoRI genomic fragment were determined by similar methods after subcloning into M13 phage. All sequences were confirmed on both DNA strands using either the appropriate universal primer or synthetic oligonucleotides (32) complementary to CK genomic or cDNA sequences. To verify the linear arrangement of the six contiguous segments of the genomic clone, DNA sequences across the restriction sites which join them were determined. To accomplish this, the intact 7.0-kb fragment was inserted into M13mp19. After template preparation, the DNA sequences surrounding the five restriction sites employed in subcloning were confirmed by deoxyribonucleotide chain termination methods and compared to those of the cDNA sequence. The restriction sites employed in subcloning were confirmed by dideoxy methods after subcloning into M13 phage. All sequences were confirmed on both DNA strands. 100 bp of 5′- and 100 bp of 3′-nontranslated region. The entire nucleotide sequence was determined (Fig. 2). The cDNA sequence corresponds exactly with nine regions containing 1598 nucleotides of coding region and 114 bp of flanking intronic sequence. It was used to identify the NH2 terminus of the predicted MtCK precursor. Total human and BC3H1 cell RNA immobilized on nylon filters as described previously (28).

DNA Sequence Analysis of MtCK cDNA—Screening 1 × 105 plaques from a human genomic library with an 800-bp dog heart MCK cDNA probe generated 15 positive signals. Southern blot analysis of one such clone, designated λhCK39, revealed that the sequences complementary to the canine MCK probe were contained entirely within a single 7.0-kb EcoRI fragment. This fragment was placed into the plasmid vector pUC13 and sequenced in its entirety (Fig. 1). Comparison of the λhCK39 nucleotide sequence with that of canine MCK cDNA revealed nine regions of extensive sequence similarity. Moreover, the λhCK39-derived amino acid sequence showed that these nine regions contained both significant similarities and differences by comparison to human MCK and BCK protein and cDNA nucleotide sequences. Therefore, we tentatively concluded that λhCK39 encodes MCK.

RESULTS

Isolation and Characterization of the Human Placental MtCK cDNA—Screening 1 × 106 plaques from a human genomic library with an 800-bp dog heart MCK cDNA probe generated 15 positive signals. Southern blot analysis of one such clone, designated λhCK39, revealed that the sequences complementary to the canine MCK probe were contained entirely within a single 7.0-kb EcoRI fragment. This fragment was placed into the plasmid vector pUC13 and sequenced in its entirety (Fig. 1). Comparison of the λhCK39 nucleotide sequence with that of canine MCK cDNA revealed nine regions of extensive sequence similarity. Moreover, the λhCK39-derived amino acid sequence showed that these nine regions contained both significant similarities and differences by comparison to human MCK and BCK protein and cDNA nucleotide sequences. Therefore, we tentatively concluded that λhCK39 encodes MCK.

A λhCK39-derived KpnI-BamHI probe, which contains 198 nucleotides of coding region and 114 bp of flanking intronic sequence, was used to screen 3 × 106 plaques from a cDNA library constructed with human placental RNA. One strong signal was generated. The 1.5-kb insert from this cDNA clone, designated pGMtCK, was isolated and the entire nucleotide sequence was determined (Fig. 2). The cDNA sequence corresponds exactly with nine regions containing 1598 nucleotides of coding region and 111 bp of flanking intronic sequence. It was used to identify the NH2 terminus of the predicted MtCK precursor. Total human and BC3H1 cell RNA immobilized on nylon filters as described previously (28).

Comparison of the cDNA nucleotide sequence with those of human MCK and BCK cDNAs (21, 22) demonstrates overall coding region identity of 62-64%. The 5′- and 3′-nontranslated regions share little similarity (<42%). Human placental MCK, encoded by pGMtCK, is distinct from MCK and BCK, yet is closely related.

The predicted amino acid sequence derived from pGMtCK allowed comparison of the degree of homology with other mammalian MCK and BCK subunits (Table I). Amino acid sequence identity among mammalian MCKs is at least 94%. Similarly, BCK sequences share an extraordinary degree of identity (95%). As we have noted previously (18), MCK and BCK within species are about 80% identical. However, when pGMtCK is compared with either human MCK or BCK cDNA-derived amino acid sequences, only 64% identity is observed (Table I and Fig. 3). Several regions of the predicted MtCK subunit sequence are highly conserved. For instance, residues 190-218, encoded by exon 5, have an identity of 83% when all known CK sequences are compared. Similarly, the region surrounding cysteine 278, the active site (36, 37), has an identity of 23 of 30 residues among all CK sequences. Conversely, the 56 NH2-terminal MtCK residues are distinctly dissimilar (identity of 26%) from cytosolic CKs. Thus, MtCK, although clearly a part of the CK gene family, is diverged significantly from the cytosolic MCK and BCK isoenzymes.

The nucleotide sequence encoding a 38-amino acid transit peptide, essential for mitochondrial targeting and import, is found at the NH2 terminus of the predicted MtCK precursor. This transit peptide functions in mitochondrial uptake, as demonstrated in Fig. 4. After in vitro transcription of the plasmid CDNA and translation of the resultant mRNA in a rabbit reticulocyte lysate, the precursor was incubated with...
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**Isolation and Characterization of the Human MtCK Gene**

Human genomic clone XhCK39 (Fig. 1) contained regions of extensive homology with MCK and BCK and was presumed to encode human MtCK. Based on its 99.99% nucleotide sequence identity with pGMtCK, its identification as the gene encoding human MtCK was established. A comparison of the nucleotide sequence of pGMtCK with this 7.0-kb genomic fragment allowed definition of the MtCK gene structure (Fig. 5, middle). The MtCK gene spans 5.5 kb, contains 9 exons, and encodes a precursor MtCK of 416 amino acids including the transit peptide. Exonic sequences range from 86-310 bp in length and are clustered in two groups (exons 1-6 and 7-9) separated by a large intron of 1.7 kb. The other 7 introns are less than 600 bp in length. The nucleotide sequences surrounding intron-exon junctions correspond well to those predicted by homology data and the AG-GT splice junction rule (38) for intron-exon boundaries (Table II).

**Comparison of Human CK NH₂-terminal Protein and cDNA-derived Sequences**

Mitochondrial and MCK were purified to homogeneity from human heart. Similarly, MCK and BCK were isolated from skeletal muscle and brain, respectively. The chemically determined NH₂-terminal sequences of these proteins are compared to the mature NH₂-terminal sequence predicted from the human placental MtCK cDNA and a recently reported rat heart MtCK protein sequence (39) in Table III. The mature NH₂ terminus of placental MtCK as predicted by comparison to the chemically determined sequence of heart MtCK. AA indicates amino acid sequence numbering which begins at the mature NH₂ terminus, with negative numbers to indicate the transit peptide. * indicates the transcription start and poly(A) addition sites.

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**Fig. 1. Complete nucleotide sequence of the human placental MtCK gene. Bold lettering indicates exons and upper case lettering indicates coding region. The nucleotide sequence is numbered along the right margin and begins with the first nucleotide encoded by genomic clone XhCK39. Predicted amino acid sequence is represented in lower case lettering.**
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MtCK residue 12 or MCK residue 17. The first 14 residues from all mature MtCK sequences shown are similar, but not identical to each other. In particular, human heart and placental MtCK share only 43% sequence identity in this region. However, residues 15-26 from all three MtCKs are identical. These data confirm that pGMtCK encodes MtCK and reveal that mature MtCK has a shortened NH$_2$ terminus as compared to MCK and BCK. Moreover, the NH$_2$-terminal differences of human heart and placental MtCK strongly suggest that the two different human MtCK mRNAs are derived from either multiple MtCK genes or alternative splicing of a single gene.

**Tissue-specific Expression of MtCK mRNA**

The $\lambda$CK39-derived Kpn-I-BaliII probe encoding sequence corresponding to mature MtCK amino acids 13-78 (exon 2, Figs. 1 and 5) detected MtCK mRNA in human adult skeletal muscle, ventricle, and to a lesser extent, small intestine and placenta (Fig. 6). No signal was detected in liver. The specificity of the MtCK gene-derived probe was demonstrated by a lack of hybridization to human MCK cDNA blotted to nitrocellulose (data not shown). The same probe detected dramatic MtCK mRNA induction in differentiating mouse BC$_3$H$_1$ muscle cells in vitro (Fig. 7). These cells are derived from a chemically induced mouse brain neoplasm. Cells were induced to differentiate by serum withdrawal and RNA was isolated over the subsequent 96 h. MtCK mRNA was first detected at 18 h after serum withdrawal and reached its greatest abundance at 48 h. The previously reported course of MCK and BCK mRNA induction during BC$_3$H$_1$ cell differentiation (13) is similar. Because the probe utilized in this experiment encodes sequence well conserved between heart and placental MtCK isoforms, we are unable to draw conclusions about the differential induction of MtCK isoforms in this system. However, these data are consistent with coordinate regulation of MtCK and cytosolic MCK expression.

**Table 1**

<table>
<thead>
<tr>
<th>Identity</th>
<th>Human Mt</th>
<th>Human M</th>
<th>Rat M</th>
<th>Rabbit M</th>
<th>Human B</th>
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**Fig. 2.** Organization, sequencing strategy and partial restriction map of the human placental MtCK cDNA. The arrows indicate the sequence determined using oligonucleotides or universal primers complementary to sequences encoded by both strands of the cDNA insert cloned in plasmid pGMtCK. The restriction map is drawn to scale. Numbering begins at the translation start site with negative numbers indicating 5'-nontranslated sequence. The hatched box indicates nucleotide sequence encoding the transit peptide, and the open box indicates sequence encoding the mature protein. The stop codon and polyadenylation (AGTAA) sites are indicated.

**Fig. 3.** Comparison of predicted human creatine kinase amino acid sequences. The amino acid sequences predicted from cDNA clones encoding MCK and BCK (21, 22) are aligned with that predicted for human placental MtCK by the cDNA, — indicates identity of amino acids among all three human gene products. * indicates identity of amino acid sequences among all known CKs in all species. The † indicates the site of intron-exon boundaries in the MCK or BCK (29) genes (R. Trask and J. Billadello, manuscript in preparation). The sites of MtCK intron-exon boundaries are indicated by a space in the sequence. % indicates the degree of sequence identity within the MtCK gene exons as compared to the corresponding cytosolic CK sequences.
To define the tissue specificity of MtCK mRNAs encoding differing isoforms, a placental-specific, pGMtCK-derived SalI-BamHI probe encoding 140 bp of 5’-untranslated region (part of exon 1, Figs. 1 and 2) was hybridized to adult human RNA (Fig. 8). The sequence encoded by this probe shares little similarity with corresponding cytosolic human CK cDNA sequences (21, 22). The probe detected MtCK mRNA from placenta and small bowel, but not from skeletal muscle, ventricle, or liver. This result conclusively demonstrates the existence of two distinct MtCK mRNAs, one found primarily in sarcomeric muscle, the other found in smooth muscle-containing tissues such as placenta and small intestine. This second species of MtCK mRNA may be ubiquitously expressed.

**DISCUSSION**

We have isolated and characterized by nucleotide sequence analysis the gene and cDNA encoding human placental mitochondrial creatine kinase. That pGMtCK encodes MtCK is verified in several ways: (i) although similar to cytosolic MCK and CCK with a nucleotide sequence identity of 64%, it is

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TABLE III  
NH2-terminal sequences of various human and mitochondrial creatine kinase subunits

<table>
<thead>
<tr>
<th>Amino acid number</th>
<th>Human placental MtCK clone</th>
<th>Human heart MtCK protein</th>
<th>Rat heart MtCK protein</th>
<th>Human MCK</th>
<th>Human BCK</th>
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<td>1</td>
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<td>N</td>
<td>C</td>
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Sequences were obtained by automated Edman degradation of intact, purified CKs. Sequences obtained only by prediction from cDNA (33) or genomic clones are shown in bold type. Rat heart MtCK protein sequence is from Cheneval and Carafoli (39). Numbering of amino acids begins with the first amino acid of mature human MtCK as the reference. The boxes demonstrate identity of amino acids among the various proteins. The splice site between exons 1 and 2 of the human MtCK gene as determined from the placental cDNA clone is indicated by the *.

Fig. 6. Tissue-specific expression of human MtCK mRNA. RNA blot hybridization analyses were performed with a 312-bp KpnI-BstII MtCK genomic fragment encoding sequence shared by heart and placental MtCK, and 5 μg each of adult total RNA from placenta, skeletal (Sk) muscle, ventricle, liver, and ileum as indicated. High stringency conditions (see “Materials and Methods”) were employed. The positions of migration of unlabeled 28 S and 18 S ribosomal RNA are indicated.

Fig. 7. Induction of MtCK mRNA in differentiating BC3H1 cells in vitro. RNA blot hybridization analyses were performed with a 312-bp KpnI-BstII MtCK genomic fragment and 10 μg of total RNA isolated from differentiating BC3H1 cells at 0, 18, 24, 48, 72, and 96 h after reduction from 20 to 2% fetal calf serum (see “Materials and Methods”). As indicated, 5 μg of total RNA from adult human (H.) liver and ventricle were included as negative and positive controls. Hybridization was performed using low stringency conditions (see “Materials and Methods”). The positions of migration of unlabeled 28 S and 18 S ribosomal RNA are indicated.

clearly a distinct protein; (ii) human heart MtCK protein residues 15-26 are identical to the corresponding sequence predicted from the placental MtCK cDNA and genomic nucleotide sequences; and (iii) the MtCK precursor is translocated into mitochondria in vitro, an event which is specific to mitochondrial protein precursors and mediated by their NH2-terminal transit peptides (35). A second major conclusion supported by our data is that at least two distinct isoforms which are tissue-specific in their expression and which contain differing transit peptides exist and are likely derived from two MtCK genes.

Comparison of the predicted placental MtCK protein sequence with sequences derived from cytosolic CK cDNAs (Fig. 3) reveals that several regions, such as the presumptive active site (Cys-283 in exon 7), are highly conserved. Other regions, which may function in the targeting and localization of MtCK to mitochondria or in interactions of MtCK with other mitochondrial proteins such as the ATP-ADP translocase (1, 24), are quite divergent from cytosolic CKs. For instance, predicted MCK, BCK, and MtCK protein sequences surrounding several MtCK intron/exon splice sites are poorly conserved (Fig. 3). In addition, NH2-terminal homology between MtCK and cytosolic CKs is low (Table III). The 25 NH2-terminal residues of mature rat heart MtCK have been identified as its cardiolipin binding domain (39). In that study, chemical modification of residues arginine 19 and lysine 20 abolished the binding of MtCK to cardiolipin. These 2 residues are absolutely conserved in all known MtCK NH2-terminal sequences (Table III), but are not present in cytosolic CKs. This finding supports the role of this domain in binding of MtCK to mitochondrial membranes. In addition, the high degree of conservation of sequence encoding residues 15-26 in heart and placental MtCK as well as the poor conservation of this region among mitochondrial and cytosolic CK isoforms are consistent with these data.

Our data (Fig. 4) reveal that the MtCK precursor is translocated into mitochondria and proteolytically processed to intermediate and mature forms. MtCK targeting thus follows the two-step pathway described for other proteins localized to the mitochondrial intermembranous space (40, 41). The
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MtCK transit peptide, 38 amino acids long, is similar in length to the transit peptide of cytochrome P-450 (42), another intermembranous space protein, and somewhat longer than those found adjoined to mitochondrial matrix proteins such as mitochondrial malate dehydrogenase (35). However, it shares the features of a typical transit peptide in that arginine and serine residues are abundant, creating a hydrophilic, positively charged structure. Preliminary sequence data from those found adjoined to mitochondrial matrix proteins such as mitochondrial transit peptide have not generally been detected, although it has been described for cytochrome c oxidase. This enzyme complex has fetal and adult isoforms (44). Southern mapping suggests that several cytochrome c oxidase subunit genes exist, and, therefore, it is likely that these isoforms are also the products of different genes (45). The pattern of isoform expression may parallel that of "embryonic" BCK and its more sarcomeric-specific counterpart, MCK.

The 5' flanking region of the placental MtCK gene is similar to previously described constitutive "housekeeping" genes such as those encoding hydroxymethylglyoxal-CoA reductase and hypoxanthine phosphoribosyltransferase (46, 47). Features common to previously described housekeeping promoters include GC rich upstream sequence, absent CAAT and TATA motifs and the existence of potential Sp1 binding sites. Upstream MtCK gene sequence has a GC content of 62%, as opposed to 48% for the entire gene, and has no AT-rich regions. The common promoter motifs, TATA and CAAT, are absent from this region (Fig. 1). Two Sp1 binding sites (CCGCC or GGGCGG) are noted at −206 and −51 from the 5' end of the placental MtCK cDNA (Fig. 1).

Several housekeeping genes are transcribed from multiple sites, although others, such as the human insulin receptor gene (48), are transcribed from one site, despite the absence of a TATA box.

Tissue-specific and developmental regulation of CK enzyme and mRNA expression has been well documented (8–11). Coupled with our studies, these data suggest that induction of MtCK mRNA is coordinated with MCK mRNA induction (13), thus providing strong experimental support of the creatine phosphate shuttle hypothesis (1, 24). For example, the steady state levels of MtCK mRNA in different tissues correlate with those of cytosolic CK mRNAs and are highest in tissues requiring high energy production, such as heart and skeletal muscle. Furthermore, we have demonstrated that, during differentiation in vitro of BC3H1 muscle-like cells, induction of high levels of expression of MtCK mRNA occurs in concert with the massive induction of MCK and BCK mRNAs. Because we have localized the placental MtCK, MCK, and BCK genes to chromosomes 15, 19, and 14, respectively (49), as yet undefined trans-acting factors are clearly necessary for this coordinated regulation of CK expression.

Acknowledgments—We are grateful to J. Billadello for graciously providing RNA from BC3H1 cells; B. C. Sobel, J. Gordon, and J. Grant for critical review of the manuscript; and H. F. Sims, R. Trask, and M. Lowe for technical assistance and moral support.

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