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Multiple isoforms and an unusual cathodic isoform of creatine kinase from channel catfish (*Ictalurus punctatus*)

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Abstract

In vertebrates, the creatine kinase (CK) family consists of two cytosolic and two mitochondrial isoforms. The two cytosolic isoforms are the muscle type (M-CK) and the brain type (B-CK). Here we report multiple CK isoenzymes in the diploid channel catfish (*Ictalurus punctatus*) with one unusual cathodic isoform that was previously found only in pathological situations in human. The cathodic CK isoform existed only in the channel catfish stomach, ovary, and spleen, but not in any other species analyzed such as tilapia, smallmouth bass, chicken, or rat. Two genes encode the multiple forms of the channel catfish M-CK cDNAs. M-CK1 has three alleles, M-CK1.1, M-CK1.2, and M-CK1.3, while M-CK2 has just one allele as determined by analysis of 17 cDNA clones and by allele-specific PCR. M-CK1 encodes a protein of 381 amino acids and the M-CK2 cDNA encodes a protein of 380 amino acids. The two cDNAs shared an 86% identity and both have the nine diagnostic boxes for cytosolic CKs and thus are of cytosolic origin. The M-CK1 gene was isolated, sequenced, and characterized and its promoter should be useful for transgenic research for muscle-specific expression.

Keywords: Creatine kinase; Polymorphism; Single nucleotide polymorphism; Marker; Expressed sequence tag; Gene

1. Introduction

Phosphorylcreatine is a high-energy phosphagen in all vertebrates. It is produced by a reversible reaction of phosphorylation of creatine by ATP catalyzed by the creatine kinases (CKs). Two fundamental types of CKs can be found in higher vertebrates, cytosolic and mitochondrial CKs, each of which has two basic isoforms. The cytosolic forms are the muscle CK (M-CK) and the brain CK (B-CK). Since CK exists as dimers, three cytosolic dimers have been found: the homodimer MM, the homodimer BB, and the heterodimer MB (Rosenberg et al., 1981; Quest et al., 1990). While there is only one form of B-CK in mammals, a second B-CK isoform has been found in chickens derived from alternative splicing of the second exon (Wirz et al., 1990). Additional heterogeneity has been found in both the M-CKs and the B-CKs. For instance, three forms of M-CK were found in humans and dogs (George et al., 1984), which were believed to be derived from hydrolytic cleavage of basic amino acids by carboxypeptidase N (Billadello et al., 1985). In chickens, alternative initiation of translation and post-translational phosphorylation led to the presence of additional isoforms (Soldati et al., 1990; Hemmer et al., 1993).

In teleost fish reported to date, four CK isoenzymes are termed CK-A to CK-D and all are of cytoplasmic origin. CK-A, CK-C, and CK-D are expressed predominantly in striated muscle, stomach, and testis, respectively, while CK-B is expressed ubiquitously or is confined to neural tissue (Fisher and Whitt, 1978). The precise number of genes encoding for the CK isozymes in teleost is not known. Three distinct cDNAs were recently cloned from a carp muscle cDNA library (Sun et al., 1998). However, since trout and carp are considered to be polyploid (Allendorf and Thorgard, 1984), such additional genes may be only a reflection of polyploidy.

In polyploid fish such as trout and carp, complex patterns of CK isozymes have been observed. Up to 13 electrophoretically distinct CKs were noted when various tissues of trout were analyzed (Scholl and Eppenberger, 1969). As a matter of fact, complex patterns of isozymes have been shown for various other enzymes in addition to the CKs in

Abbreviations: ATP, adenosine triphosphate; CK, creatine kinase; B-CK, brain creatine kinase; M-CK, muscle creatine kinase; EST, expressed sequence tag; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; UTR, untranslated region

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polyploid fish (Massaro and Markert, 1968; Bailey et al., 1969). Additionally, some CK isoforms in trout also were believed to be from epigenetic modifications or conformational isozymes (Scholl and Eppenberger, 1969).

Although complex patterns of isozymes are common in polyploid fish, there are no reports of multiple CK isoforms in diploid fish. Here we report multiple isoforms of CK in channel catfish (*Ictalurus punctatus*) and a highly unusual cathodic isoform present in the channel catfish stomach, ovary, and spleen. The cathodic CK isoform has only been detected in malignancies or other serious pathological conditions in humans (Miyake et al., 1980). In this work, we found that the cathodic isoform was present only in channel catfish, but not in stomach tissue of tilapia (*Oreochromis niloticus*), smallmouth bass (*Micropterus dolomieui*), chicken, or rat. Multiple M-CK cDNAs were characterized and the M-CK1 gene encoding the abundant CK transcripts was isolated and sequenced.

2. Materials and methods

2.1. RNA isolation and construction of cDNA libraries

Experimental fish were raised in troughs located in the hatchery of the Auburn University Fish Genetics Facility. Fish were reared under the same conditions for 4 weeks before the tissues were harvested. Muscle tissue was collected from channel catfish for preparation of total RNA. Muscle tissue was frozen in liquid nitrogen, ground with a mortar/pestle and then homogenized with a hand-held tissue tearor (Model 985-370, Biospec Products, Inc., Bartlesville, OK) in RNA extraction buffer following the guanidium thiocyanate method (Chomczynski and Sacchi, 1987). Poly $(A)^+$ RNA was then purified by using the Oligotex Spin Column Kit from Qiagen according to the manufacturer's instructions (Qiagen Inc., Chatsworth, CA). The muscle cDNA library was constructed using the UNIZAP cloning system (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

2.2. Library screening and isolation of the channel catfish M-CK1 gene

A channel catfish genomic DNA library was custom-made by Stratagene using genomic DNA isolated from a male channel catfish. The genomic DNA was digested by *Sau*3A1 and cloned into the *Bam*H1 site of the Lambda Dash cloning vector. The library was screened using channel catfish M-CK1.1 cDNA probe labeled using [^{32}P]dCTP. A single recombinant clone was identified by repeated plaque purification (Sambrook et al., 1989). Restriction mapping and partial sequencing were used for initial characterization of the clone. The fragments containing the channel catfish M-CK1 gene were subcloned for sequencing analysis (Sambrook et al., 1989).

2.3. DNA sequencing and sequence analysis

Nucleotide sequences were determined by the dideoxyribonucleotide chain termination method using the LI-COR automated DNA sequencer. Mini-preparation plasmid DNA (1 μl, about 200–500 ng) was used for all sequencing reactions using a thermosequenase kit (Amersham, Piscataway, NJ). The profiles for cycling were: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles. An initial 2 min of denaturation at 94°C was always used. Complete sequences of the cDNAs or the gene were obtained by primer walking sequencing. Typically, approximately 600–800 base pairs (bp) of sequences were obtained per run, but primers were designed every 400–450 bp to assure accurate reading. DNA sequences were analyzed using the DNASIS and DNASTAR software packages. Homology searches were conducted using the BLASTN program of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).

For expressed sequence tag (EST) analysis, channel catfish M-CK cDNA clones obtained in the process of EST analysis were sequenced. Altogether 17 clones of the channel catfish CKs were analyzed.

2.4. Southern blot analysis

Genomic DNA was first digested with restriction endonuclease *Eco*RI, *Eco*RV, and *Pst*I separately and electrophoresed on a 0.8% agarose gel (Southern, 1975). The DNA was transferred to a piece of Zetabind nylon membrane (Whatman, Madison, WI) by capillary transfer with 0.4 M NaOH overnight (Reed and Mann, 1985). Membranes were dried at 80°C under vacuum for 2 h. The membrane was washed in 0.5% SDS (w/v) at 65°C for 15 min and then pre-hybridized in 50% formamide, 5× SSPE (Sambrook et al., 1989), 0.1% SDS (w/v), 5× Denhardt's and 100 μg/ml sonicated and denatured calf thymus DNA overnight. Hybridization was conducted overnight at 42°C in the same solution with probes added (greater than 10^9 counts/min per μg DNA). The M-CK1.1 cDNA was used as a probe for the Southern blot. The probe was prepared using the random primer method (Sambrook et al., 1989) with a labeling kit from Boehringer Mannheim (Indianapolis, IN). The Zetabind membranes were washed first in 500 ml of 2× SSC for 10 min, followed by three washes in 0.2× SSC with SDS at 0.2% (w/v) at 65°C for 15 min each. The membranes were then wrapped in Saran wrap and exposed to Kodak BioMax MS film for autoradiography.

2.5. Sequence-specific PCR analysis

We used a sequence-specific PCR analysis to determine if a specific sequence existed in an individual fish. M-CK cDNA sequences were aligned to find the single nucleotide polymorphism (SNP) sites. PCR primers were designed at the SNP site with each to match its own sequences and,

therefore, unable to amplify the alternative sequence. To make this sequence-specific PCR analysis more reliable, we conducted two PCR reactions in the same test tube. In this design, the lower primer was shared by the two PCR reactions and the two upper primers each could only amplify its own specific sequences. The upper primers were differentially labeled with IR700 and IR800 fluorescence dye, respectively. Their specific PCR products were detected using the LI-COR automated sequencer.

2.6. CK isozyme analysis

The following tissues were collected from channel catfish: muscle, gill, liver, heart, spleen, head kidney, trunk kidney, stomach, intestine, adipose, ovary, testis, brain, and eye. Stomachs were also collected from tilapia and smallmouth bass. Chicken stomachs were kindly provided by Dr. Edward Moran, Department of Poultry Science, Auburn University. Rat stomachs were kindly provided by Dr. Siyami Karahan, College of Veterinary Medicine, Auburn University. In all cases stomach contents were removed completely and the stomachs were rinsed well before the tissue was frozen. Tissues were frozen in liquid nitrogen and stored at −80°C before analysis of CK activity. Each tissue was dissected and homogenized in an equal volume (w/v) of chilled extraction buffer (0.25 M Tris, pH 7.0) on ice with a motor-driven Teflon pestle. The resultant homogenate was centrifuged at $3000 \times g$ for 3 min in a Beckman benchtop centrifuge and the supernatant was collected and subjected to starch gel electrophoresis.

Horizontal starch gel electrophoresis was performed according to standard protocol (Shaklee et al., 1973) at 4°C for 5 h at 200 V in 11% starch gels in a pH 8.1 continuous R buffer system. The R buffer system is used routinely to resolve CK isozymes in our laboratory. The stock pH 8.1 R buffer contained 0.06 M lithium hydroxide and 0.3 M boric acid. A 1:6 dilution of this stock buffer plus 0.03 M Tris and 0.005 M citrate was used for making gels. Following electrophoresis, the gels were sliced horizontally and stained for CK activity by incubating them in an appropriate amount of staining solution: 10 ml R buffer, 150 mg phosphocreatine, 15 mg adenosine 5′-diphosphate, 10 mg β-nicotinamide adenine dinucleotide phosphate, 100 units hexokinase, 20 mg α-D-glucose, 10 mg $MgCl_2$, 30 units of glucose-6-phosphate dehydrogenase, 10 mg MTT, and 3 mg phenazine methosulfate (Sigma, St. Louis, MO).

3. Results and discussion

3.1. Multiple isoforms of CK enzyme in tissues of channel catfish

Seven distinct isoforms of CK enzyme activity were detected by electrophoretic analysis of the 14 channel catfish tissues, six of which were anodal while the seventh was cathodic (Fig. 1). In most tissues, more than one isoenzyme type was found. As expected, no CK activity was found in adipose tissue. High CK enzyme activity was detected in muscle, liver, stomach, brain, spleen, heart, and ovary, and low CK enzyme activity was detected in trunk kidney and intestine. The highest CK activity was detected in muscle as reflected by the strong signals from the enzymatic staining deposit (Fig. 1).

An unusual cathodic isoform was detected from the channel catfish stomach, ovary, and spleen (Fig. 1). The cathodic CK isoenzyme was expressed at a high level in stomach (Fig. 1). Lower levels of the cathodic CK isoform were found in ovary and spleen, which could be detected only with extended enzymatic staining. The cathodic CK isoforms from stomach, ovary, and spleen appeared to have a similar pattern and migration in starch gels, but they may not necessarily be identical because the distance migrated from the origin was relatively short and, therefore, the resolution was limited. Among the six anodal isoforms of the channel catfish CKs, the migrating distances from the origin were dramatically different suggesting significant charge differences among the CK isozyme.

3.2. Specific presence of the cathodic isoforms of CK in channel catfish

One possibility for the cathodic CK isoforms could be the special pH environment in the stomach and ovaries. If this was true, one would expect similar cathodic CK from stomachs of other species as well. To determine if the cathodic isoform of the CK could be an artifact produced by the low pH environment in the stomach, stomach tissues were collected from four more vertebrate species ranging from fish and birds to mammals. Clearly, only channel catfish has the cathodic CK isoenzyme (Fig. 2). Only anodal isoforms were detected in tilapia, smallmouth bass, chicken, and rat.

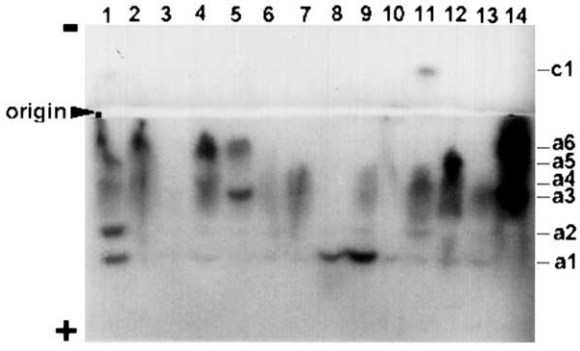

Fig. 1. Multiple isoforms of the channel catfish CK. At least seven isoenzymes were detected as marked on the right margin: six anodal isoforms are indicated by a1–a6 and one cathodic isoform is indicated by c1. Tissue homogenates were loaded at the origin (arrow) and electrophoresed on a starch gel in a pH 8.1 continuous R buffer system with electrode direction indicated on the left margin: +, cathode; −, anode. Fourteen samples were loaded as follows: ovary (lane 1), testis (lane 2), adipose (lane 3), spleen (lane 4), heart (lane 5), trunk kidney (lane 6), head kidney (lane 7), eye (lane 8), brain (lane 9), intestine (lane 10), stomach (lane 11), liver (lane 12), gill (lane 13), and muscle (lane 14).

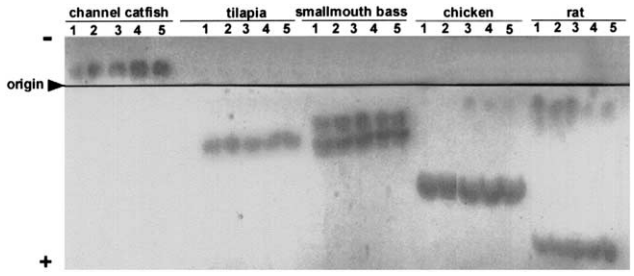

Fig. 2. Analysis for the presence of the cathodic CK in stomachs of channel catfish, tilapia, smallmouth bass, chicken, and rat. Five individuals (1–5) were used for each species. Stomach homogenates were loaded at the origin (arrow) and electrophoresed on a starch gel in a pH 8.1 continuous R buffer system with electrode direction indicated on the left margin: +, cathode; −, anode.

No individual polymorphism was detected in any of the stomach tissues examined. Five individuals each of the channel catfish, tilapia, chicken, and rat all exhibited the same isozyme patterns in their stomachs (Fig. 2).

To confirm that the cathodic CK enzyme was not an artifact caused by the buffer system, we also used other buffers with both acidic and basic pH for analysis of the catfish stomach CK. The buffers used include buffer A (pH 7.0, 0.13 M Tris, 0.045 M citrate, and 0.0012 M EDTA), buffer M (pH 8.7, 0.18 M Tris, 0.1 M boric acid, and 0.004 M EDTA), and buffer 4 (pH 6.3, 0.223 M Tris and 0.094 M citric acid). Electrophoresis of fresh stomach homogenates of channel catfish using these buffers produced the same results as with buffer R (data not shown) suggesting that this cathodic isoenzyme is not a buffer-induced artifact. Thus, the cathodic CK enzyme appeared to exist only in channel catfish. However, the assays we used were enzyme coupling assays. The assumed reactions can also be catalyzed by adenylate kinase or guanylate kinase. To determine if the cathodic form could have been the products of these alternative enzymes, we performed assays for guanylate kinase and adenylate kinase using the same samples and under the same conditions (Manchenko, 1994). The results revealed that the cathodic band was produced only in the CK assay, but not in the guanylate kinase or adenylate kinase reactions (data not shown).

Elevated expression of B-CK and subsequent release into serum has been used as a diagnostic marker of several types of malignancies involving solid tumors in humans (Wallimann and Hemmer, 1994). In channel catfish, two B-CK isoforms existed with the fastest and the slowest migration in starch gels among the anodal isoforms. During pathological insults and development, the human CK has been found to shift toward the B isoform in cardiac, skeletal, and smooth muscles. However, cathodic CK forms are highly unusual in humans. Limited cases of cathodic CK were found only in patients with stomach cancer (Miyake et al., 1980), breast cancer and skin metastasis (Yuu et al., 1978), and with Rey's syndrome (Rock et al., 1975). After a survey of CK in stomachs of 16 fish species, Scholl and Eppenberger (1972) did not detect any cathodic bands. The physiological significance of the cathodic CK enzyme in channel catfish is unknown at present.

3.3. Multiple forms of M-CK cDNAs from channel catfish

The multiple isoforms, particularly the presence of the cathodic isoform in channel catfish, indicated that the channel catfish CKs may be encoded by a multigene family, and/or they may be subjected to post-transcriptional processing such as differential splicing (Wirz et al., 1990) and/or extensive post-translational modifications. We were particularly interested in analyzing as many muscle cDNA clones of the channel catfish CK as possible. In an analysis of muscle ESTs, 17 clones of CK were found. Nucleotide sequence analysis of the 17 clones revealed four different CK cDNA sequences. Three of the four cDNAs were almost identical differing only in a couple of nucleotides (Table 1). We suspected that these CK cDNAs could be derived from SNP in different individuals since the channel catfish muscle cDNA library was constructed from muscle tissues of multiple individuals. These three most similar CK cDNAs were designated as M-CK1.1, M-CK1.2, and M-CK1.3. Sequences of M-CK1.1 and M-CK1.2 encode identical CK proteins and the M-CK1.3 encodes a CK protein with only one amino acid substitution (aspartic acid to alanine) as compared to M-CK1.1 and M-CK1.2 (Table 1). The fourth cDNA is quite distinct with 86% nucleotide similarity to the other three M-CK cDNAs and was referred to as M-CK2 (Fig. 3). The channel catfish M-CK1.1, M-CK1.2, and M-CK1.3 encode a protein of 381 amino acids, while the channel catfish M-CK2 encodes a protein of 380 amino acids.

Three types of M-CK cDNA were reported from carp (Sun et al., 1998). The channel catfish M-CK1.1, M-CK1.2, and M-CK1.3 are most similar to the carp M1-CK and M2-CK, but are divergent from the carp M3-CK. In contrast, the channel catfish M-CK2 is more similar to the carp M3-CK. The carp M3-CK is reported to exhibit peak activity at 25°C (Sun et al., 1998). Since the channel catfish M-CK2 is similar to the carp M3-CK, it is likely that the channel catfish M-CK2 could be the functional equivalent of

Table 1
Comparison of M-CK1.2 and M-CK1.3 cDNAs with M-CK1.1[a]

M-CK1.1	Position 50	Position 732	Position 1449	Position 1451
M-CK1.2	nt: A → C; aa: D → A	nt: A → C; aa: no change	nt: A → C; located in 3′ UTR	nt: A → T; located in 3′ UTR
M-CK1.3		nt: A → C; aa: no change		

[a] Coordinates are nucleotide positions from the translation initiation site ATG of M-CK1.1.

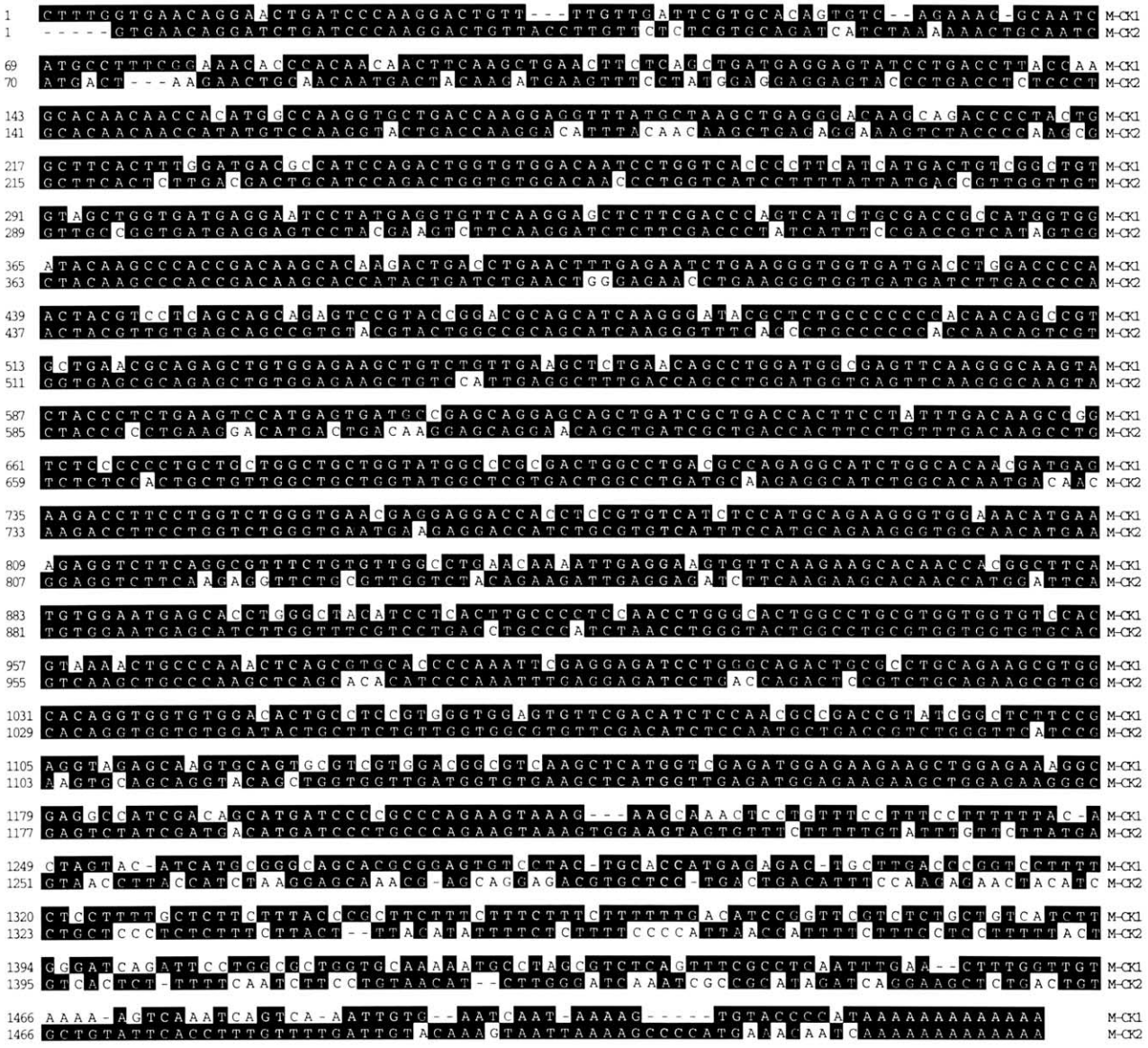

Fig. 3. cDNA sequence comparison of the channel catfish M-CK1 and M-CK2. Black boxes are identical and white boxes are divergent nucleotides between the two cDNAs. Dashes (–) indicate deletions.

M3-CK being more active at lower environmental temperatures. In this sense, both carp and channel catfish harbor dual forms of M-CK genes for specific physiological requirements.

The channel catfish M-CK1.1 and M-CK2 cDNAs shared 86% nucleotide sequence identity (Fig. 3). The M-CK1.1 consisted of an 1146 bp coding region, a 72 bp 5′ non-translated region (NTR), and a 300 bp 3′ NTR. The cDNA of M-CK2 consisted of an 1143 bp coding region, a 69 bp 5′ NTR, and a 310 bp 3′ NTR. A typical polyadenylation signal, AATAAA, is located 18 bp upstream of the poly $(A)^+$ sequences in M-CK1.1. In M-CK2, a variation of the poly $(A)^+$ signal sequence ATTAAA existed 24 bp upstream from the poly $(A)^+$ sequences. This variation of the poly A signal sequences in the M-CK2 gene is identical to that in the carp M3-CK gene (Sun et al., 1998) and that of the gonadotropin beta-I gene of catfish (Liu, unpublished data) suggesting that ATTAAA is widely used as a polyadenylation signal in teleost fish genes. The channel catfish M-CK coding regions are highly conserved as compared to other CK genes (data not shown), particularly at the six highly conserved domains (Muhlebach et al., 1994). All nine diagnostic boxes characteristic of the cytosolic CK genes existed in the channel catfish M-CKs suggesting that all the CK cDNAs were from cytosolic CKs, not mitochondrial CKs.

3.4. Two genes encode the abundant M-CK mRNAs

The most abundant representation of M-CK in the channel catfish muscle cDNA library indicated either that the M-CK was encoded by a multigene family or the M-CK gene

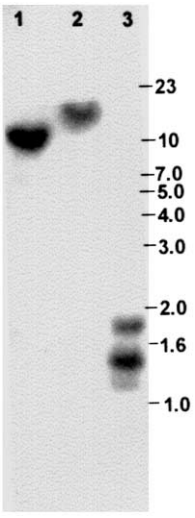

Fig. 4. Southern blot analysis of the channel catfish M-CK. Channel catfish genomic DNA was digested with *Eco*RI (lane 1), *Eco*RV (lane 2), and *Pst*I (lane 3) and blotted to nylon membrane and hybridized to the channel catfish M-CK1.1 cDNA probe. Molecular weight standards are indicated on the right margin.

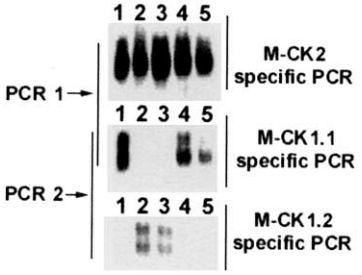

Fig. 6. Results of sequence-specific, three-primer PCR showing that M-CK2 exists in all individual fish tested, while in a given fish, either the M-CK1.1 or the M-CK1.2 existed. Five fish were tested (lanes 1–5) in two PCR reactions as shown on the left margin (PCR 1 and PCR 2). In PCR 1, the three primers used were a common lower primer for M-CK1 and M-CK2, a M-CK1.1-specific upper primer, and a M-CK2-specific upper primer. PCR products from the M-CK2-specific primer were detected with an IR800 channel (top) and PCR products from M-CK1.1-specific primer were detected with an IR700 channel (middle) in PCR 1. Similarly, in PCR 2, the three PCR primers used were the common lower primer, the M-CK1.1-specific upper primer, and a M-CK1.2-specific upper primer whose PCR products were detected with an IR800 channel.

harbors a strong promoter. If it is the latter, the M-CK promoter may be a potentially valuable resource for transgenic fish research for high-level, muscle-specific gene expression. Although four different cDNA sequences were found, three of the four were almost identical and may be the transcript of a single gene with SNP in different individuals. As the first step in determining the M-CK gene copies in channel catfish, a genomic Southern blot was performed. As shown in Fig. 4, a single band was detected when the genomic DNA was digested with restriction endonuclease *Eco*RI or *Eco*RV, while three bands were detected when the genomic DNA was digested with restriction endonuclease *Pst*I. Thus, the Southern blot analysis indicated that the M-CK gene could exist as a single gene since the two restriction endonucleases produced a single band. Supporting this notion is the presence of three small fragments (<2 kb) when digested with restriction endonuclease *Pst*I suggesting the presence of more *Pst*I sites within the M-CK gene in addition to the one found in the cDNA sequences. However, the single gene hypothesis does not account for the multiple forms of M-CK mRNAs as revealed by cDNA sequencing analysis, especially the highly divergent M-CK2. As discussed above, the M-CK1 and M-CK2 share 86% identity. A 14% divergence was too high to be accounted for by SNP variation among individuals. As a matter of fact, SNP rates in channel catfish were lower than 1% in coding regions of several hundred ESTs that we analyzed (Liu, unpublished data).

Alternative approaches were then considered to determine how many genes were involved in transcription of the four different M-CK cDNA types. One approach was to determine if all four cDNAs could be found in the same individual fish. To accomplish this, we have used a sequence-specific 'three-primer PCR' analysis (Fig. 5). In the sequence-specific, three-primer PCR procedure for SNP detection, three primers were used for PCR; one of the PCR primers was designed to prime PCR reactions for both sequences and the other two primers each was designed to

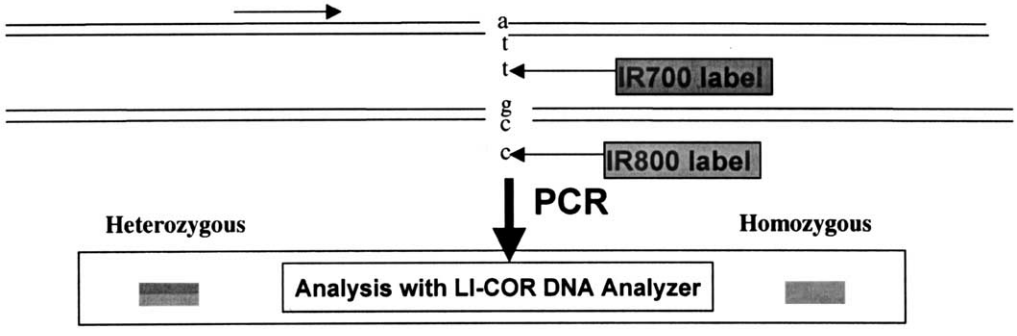

Fig. 5. Schematic presentation of the sequence-specific three-primer PCR. Three PCR primers were used with the upper primer (arrow) being common for both sequences. Lower primers were sequence-specific and labeled differentially (IR700 and IR800, respectively). After PCR amplification, PCR products were analyzed on a LI-COR automatic DNA sequencer.

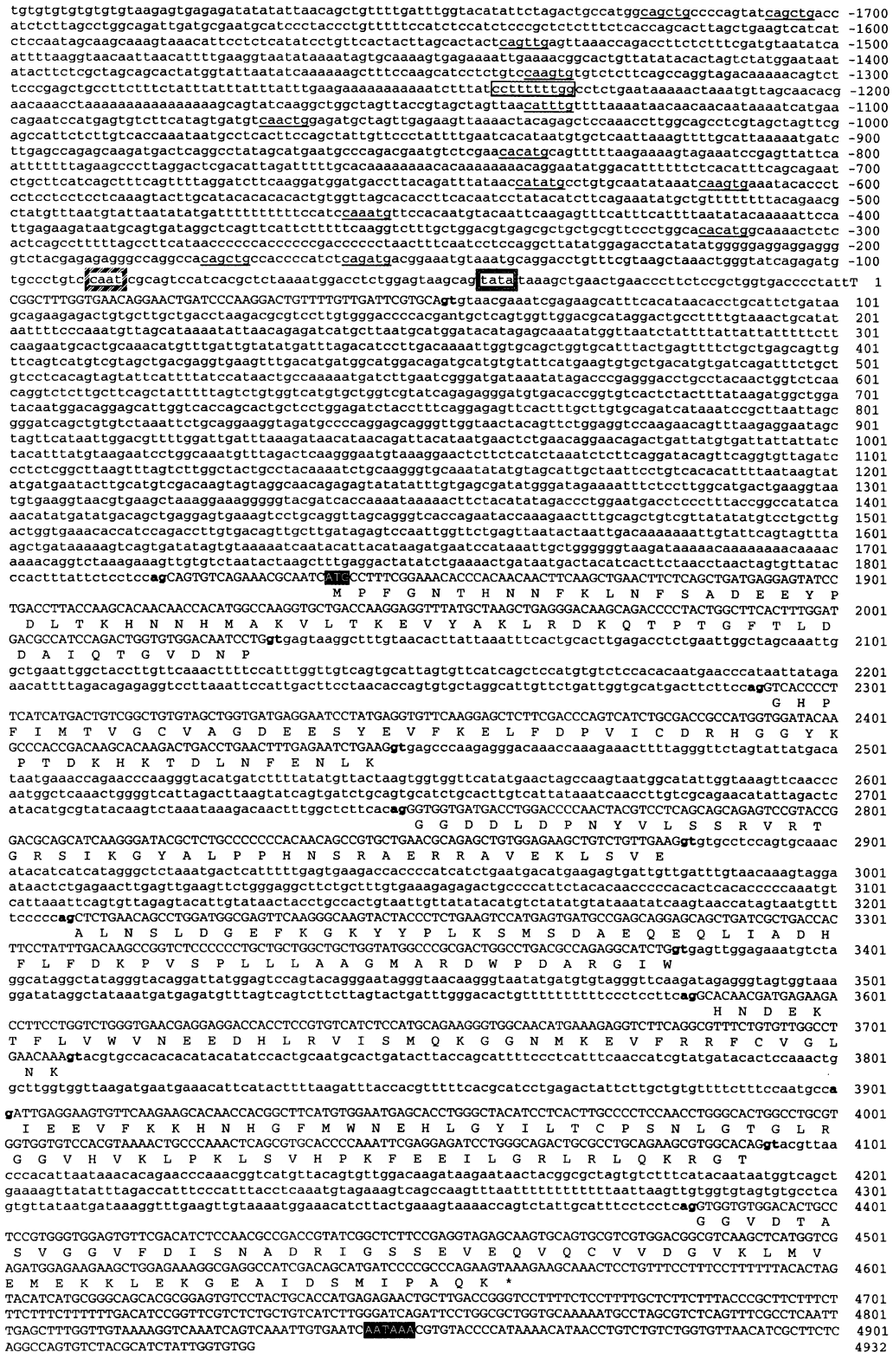

Fig. 7. The nucleotide sequences of the channel catfish M-CK1 gene. Nucleotides are numbered relative to the transcription initiation site at +1. The exons are shown in upper case letters and the introns are in lower case letters. The deduced amino acid sequences are indicated below the nucleotide sequences in the one-letter amino acids code. The putative transcriptional regulatory motifs are indicated including E-boxes (underlined), CAAT, TATA, and CArG boxes (respective boxes). Translational start codon ATG and the polyadenylation signals AATAAA are indicated by a dark background. Splicing junctions are indicated by bold letters.

prime PCR reactions only for its own sequence because the last base at the 3′ of the primer is sequence-specific and derived from the SNP site. The two sequence-specific primers were labeled by infrared-700 nm (IR700) and infrared-800 nm (IR800) fluorescence dye, respectively. PCR products were detected by analysis of the products using a LI-COR automated sequencer with laser detectors for both IR700 and IR800. If a single fish harbors only one sequence, PCR products can only be detected at one laser channel of the sequencer. In contrast, if a single fish harbors both sequences, PCR products can be detected from both laser channels of the sequencer. Four cDNA sequences were aligned to identify DNA sites containing SNP. At the SNP site, a locus-specific, three-primer PCR analysis was conducted to determine if only one or both polymorphic SNP existed in a single individual fish. The presence of only one or the other SNP in a single individual fish indicated that the SNP at this site of cDNA was derived from individual polymorphism in the population, while the presence of both SNPs in a single individual indicated the presence of two genes. As shown in Fig. 6, M-CK2-specific primer produced PCR products in all five fish tested, while either M-CK1.1-specific or M-CK1.2-specific bands were produced, indicating that the sequence variations between M-CK1.1 and M-CK1.2 represent allelic variations in the populations. Similar results were obtained with M-CK1.3-specific primers (data not shown). These results confirm that two M-CK genes exist in channel catfish. Consistent with this result was the frequency of each cDNA found among the 17 clones sequenced. Eight M-CK1 clones (including M-CK1.1, M-CK1.2, and M-CK1.3) and nine M-CK2 clones were found.

Taken together with the previously presented Southern blot analysis, these data suggested that the two copies of the M-CK genes were derived from gene duplication surrounding the CK gene region followed by nucleotide substitutions in evolution. Therefore, herein the CK genes will be referred to as M-CK1 and M-CK2. In other words, in any given channel catfish, M-CK has two genes. The M-CK1.1, M-CK1.2, and M-CK1.3 represented allelic SNP among individuals.

3.5. Sequence analysis of the M-CK1 gene

The abundant M-CK mRNAs were encoded by only two genes suggesting that their promoters are strong. To isolate the CK gene promoter, the M-CK1 gene was isolated by screening a genomic DNA library by using a ^{32}P-labeled channel catfish M-CK1.1 cDNA probe. After three rounds of screening, one clone, λ7, was purified containing the CK homologous sequences on genomic DNA fragments of approximately 11.4 kb. Restriction mapping of this clone indicated that the CK gene was included within a 7.2 kb fragment. The complete nucleotide sequences of the M-CK1 gene are shown in Fig. 7. The overall structure of the channel catfish M-CK1 gene is similar to CK genes from other species with eight exons (e.g. Benfield et al., 1988). The channel catfish M-CK1 gene is approximately 6.7 kb long including the 1.8 kb upstream sequences. The nucleotide sequences surrounding all intron–exon donor and acceptor junctions conform to the AG-GT splice consensus rules. The exon–intron sequences were determined by comparison of the genomic sequences with the cDNA sequences. The non-translated first exon is conserved in the channel catfish M-CK1 gene with a 1.7 kb intron before the translational initiation codon ATG within the second exon (Benfield et al., 1988; Klein et al., 1991; Muhlebach et al., 1996).

Sequence analysis indicated the presence of a number of important transcriptional regulatory elements (Fig. 7). The presumed transcriptional initiation site was tentatively determined by the start of the analysis of the 17 M-CK cDNA clones. Within the sequenced 1.8 kb upstream sequences, many putative transcriptional regulatory motifs existed in the M-CK1 gene including the CAAT box, the TATA box, 13 E-boxes (Davis et al., 1990), and one CArG box (Miwa et al., 1987). Further analysis of promoters and transcriptional elements is underway in our laboratory. The availability of strong muscle-specific promoter should be valuable for directing tissue-specific gene expression in muscles.

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