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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 272, No. 28, Issue of July 11, pp. 17790–17794, 1997
Printed in U.S.A.

Functional Equivalence of Creatine Kinase Isoforms in Mouse Skeletal Muscle*

(Received for publication, April 1, 1997, and in revised form, May 7, 1997)

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Creatine kinase (CK) is a highly conserved enzyme abundant in skeletal muscle that has a key role in high energy phosphate metabolism. The localization of the muscle isoenzyme of CK (MM-CK) to the M line and the sarcoplasmic reticulum of myofibrils has been suggested to be important for proper force development in skeletal muscle. The importance of this subcellular compartmentation has not been directly tested *in vivo*. To test the role of myofibrilar localization of CK, the consequences of a complete CK isoform switch from MM-CK to the brain (BB-CK) isoform, which does not localize to the M line, was studied in transgenic mouse skeletal muscle. In MM-CK knockout mice there are large contractile defects. When MM-CK was replaced by BB-CK, the aberrant contractile phenotypes seen in MM-CK knockout mice were returned to normal despite the lack of myofibrillar localization. These results indicate that CK compartmentation to the myofibril of skeletal muscle is not essential for contractile function and that there is functional equivalence of creatine kinase isoforms in supporting cellular energy metabolism.

There are a growing number of examples of subcellular compartmentation of metabolic enzymes. Many of the enzymes of glycolysis in addition to being in the cytosol localize to subcellular structures such as the outer membrane of mitochondria, actin, the sarcoplasmic reticulum, and the plasma membrane (1, 2). Krebs cycle enzymes associate into a metabolon that is believed to aid the shuttling of substrates from enzyme to enzyme in the crowded matrix space of the mitochondrion (3). The enzymes involved in nucleotide synthesis are organized to allow efficient channeling of substrates (4–6). Some tRNA synthetases associate into high molecular weight complexes, and evidence indicates that tRNAs are channeled (7–9). There is ample theoretical work that indicates that subcellular compartmentation of enzymes affords advantages for metabolic control and a large amount of *in vitro* and *in vivo* evidence exists indicating that subcellular compartmentation alters enzymatic activity and substrate utilization (10–12). Despite all this work, no consensus has developed concerning whether or not subcellular compartmentation is important for maintaining mammalian cell and tissue function *in vivo*.

Creatine kinase (CK)[1] is central to cellular energy metabolism and is one of the best studied examples of an enzyme that exhibits subcellular compartmentation (13–16). CK catalyzes the following reaction: phosphocreatine (PCr) + ADP + H^+ = creatine + ATP. CK exists in several isoforms that differ in their developmental and tissue patterns of expression and subcellular localization (16). The CK catalyzed reaction plays an important role in maintaining ATP levels and modulating intracellular pH during periods when utilization of ATP exceeds production by glycolysis or oxidative phosphorylation. In addition, PCr represents a significant store of inorganic phosphate. The muscle isoenzyme of CK (MM-CK) is abundant in the cytoplasm and associated tightly with the M line of myofibrils and the outer face of the sarcoplasmic reticulum membrane, and in avian skeletal muscle, it is associated with the I-band (17–20). *In vitro* evidence indicates that localization to these sites is important for force generation and calcium pumping (21–27). The brain isoform of CK (BB-CK) is also found in the cytoplasm and has been shown to localize to plasma membranes in the vicinity of the Na-K ATPase and mitotic spindles (28, 29). MM-CK and BB-CK are dimers formed from subunits that can also associate to form a muscle-brain (MB) heterodimer that is found in heart. In addition to the cytosolic forms, there are two mitochondrial forms of CK, sarcomeric and ubiquitous mitochondrial CK, which localize to the intermitochondrial membrane space and have been implicated in affecting regulation of oxidative phosphorylation (13–16). The localization of CK to sites of ATP production and utilization has led to the proposal that CK plays a role in spatial buffering of ATP, ADP, and pH that is important for proper tissue function and in particular, maintaining contractile function in muscle and heart (13–16).

Inhibition of CK in muscle using substrate analogues (30–32) or gene knockout of MM-CK and mitochondrial CK (33–36) has been shown to lead to large contractile abnormalities and alterations in muscle cell size and alterations in mitochondrial and glycolytic capacity. These results clearly demonstrate the important role of CK in normal muscle function but do not specifically address whether the CK isoform expression that is localized to myofibrils plays an important role in contractile function. Previously, a mouse expressing the B subunit of CK (B-CK) in skeletal muscle in addition to wild-type M subunits

* This work was supported by Grant-in-Aid 94017810 from the American Heart Association with funds contributed in part by the Pennsylvania Affiliate of the American Heart Association, by a National Institutes of Health Research Career Development Award 5 K04 HL-02847, by National Institutes of Health Grant R01-HL40354 (to A. P. K.), and by a National Institutes of Health National Research Service Award F32-HL08985 (to B. B. R.). The Pittsburgh NMR Center for Biomedical Research is supported by National Institutes of Health Grant RR-03631. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CK, creatine kinase; PCr, phosphocreatine; MM-CK, muscle isoenzyme of CK; BB-CK, brain isoform of CK; B-CK, B subunit of CK; M-CK, M subunit of CK; M-CK KO, mice that lack M-CK subunits due to gene disruption.

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(M-CK) has been described (37, 38). These mice show normal contractile activity with no evidence of adaptation. Furthermore, it was demonstrated that BB-CK does not localize to myofibrils. The availability of the mice expressing B-CK subunits and mice that lack M-CK subunits due to gene disruption (M-CK KO) enabled generation of a mouse with skeletal muscle containing a complete isoenzyme switch from MM-CK to BB-CK. Thus, a direct test of whether specific localization of CK to myofibrils is essential for proper function of muscle was performed.

EXPERIMENTAL PROCEDURES

Animal Care—All animals were bred and maintained in groups of four in a temperature-controlled (25 °C) room. Agway rat chow (Rat-mouse-hamster 3000, Agway Co.) and water were provided *ad libitum*. All animals used were male and 8–16 weeks of age. Animals were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), and the initial anesthetic dose lasted for the duration of the experiments. All protocols involving animals were approved by the Institutional Animal Care and Use Committee.

Expression of B-CK in Skeletal Muscle—Production of transgenic mice that express the B subunit of CK in skeletal muscle (7001 line) was described previously (37, 38). Mice heterozygous for a B-CK transgene and homozygous for M-CK KO allele were generated by mating M-CK KO mice (33–35) with the 7001 line of B-CK overexpressing mice (37, 38) and the offspring back-crossed to the M-CK KO. These mice were then intercrossed to create mice homozygous for the B-CK transgene and homozygous for the M-CK KO allele (referred to as switch mice).

Biochemical and Immunoflourescence Assays—The left superficial gastrocnemius muscle was isolated for chemical extraction (for glycogen assessment the entire gastrocnemius was used). All biochemical assays and extractions were performed using standard techniques as described previously (38). Tissue sections were immunoassayed using goat anti-human CK polyclonal primary antibodies (Cortex, Inc., CA) and Cy3 conjugated secondary antibody. 4-μm slices were prepared from muscles that were isolated, frozen in liquid nitrogen cooled isopentane, and mounted for cutting using TBS-tissue freezing medium (Fisher). After sectioning, the tissue was then incubated with primary antibody (1:300 dilution/10% horse serum) for 2 h at room temperature in a humid chamber. Sections were washed off with 1 × phosphate-buffered saline/10% horse serum. The secondary antibody (1:500/10% horse serum) conjugated to Cy3 was applied for 30 min at room temperature. The sections were washed and coverslips were applied. Isolated myofibrils were washed and assayed for CK as described previously (37).

Mechanical Function—The animals were anesthetized and prepared for stimulation as described previously (38). Muscles were stimulated via platinum electrodes attached to the sciatic nerve, and force measurements were made using a calibrated F-100 transducer (MacLab). The force records were recorded using a MacLab Data Acquisition system. Rest tension was applied to the muscle and adjusted following test twitches until an increase in force was no longer observed with constant voltage. The muscle was allowed to recover for approximately 10 min following the test contractions. Muscles were supramaximally stimulated at 5 Hz, and force was recorded for 3 min.

NMR Data Acquisition—Anesthetized animals were in a supine position within the magnet bore. ^{31}P NMR spectra were acquired at 121.55 MHz on a Bruker Biospec 7T spectrometer. A two-turn rectangular surface coil that was approximately the size of the gastrocnemius-plantaris-soleus complex was used. The coil was tuned to the phosphorus signal and shimmed on the proton signal. Phosphorus spectra were obtained using a 90 ° flip angle (40 μs square pulse), a 4K data set, and a 15 s repetition delay. Magnetization transfer was measured using a saturation transfer pulse sequence and irradiation positioning on the γ-ATP resonance as well as a control position as described previously (37). Saturation and control irradiation times between 0.5–14.5 s were used. Peak integration of the PCr peak was used to measure signal intensity, and the data were fit to the following exponential function: $M_t/M_0 = 1 - k_{for}\,T_{1\,app}\,(1 - \exp(-t/T_{1\,app}))$, where M_t/M_0 is the PCr signal intensity during γ-ATP and control saturation, k_{for} is the first order rate constant, t is the duration of the saturation, and $T_{1\,app}$ is the T_1 apparent time constant.

Statistics—The values represented in the text, tables, and figures are the sample means ± S.D. Significance was accepted at $p < 0.05$ using an unpaired Student's t test.

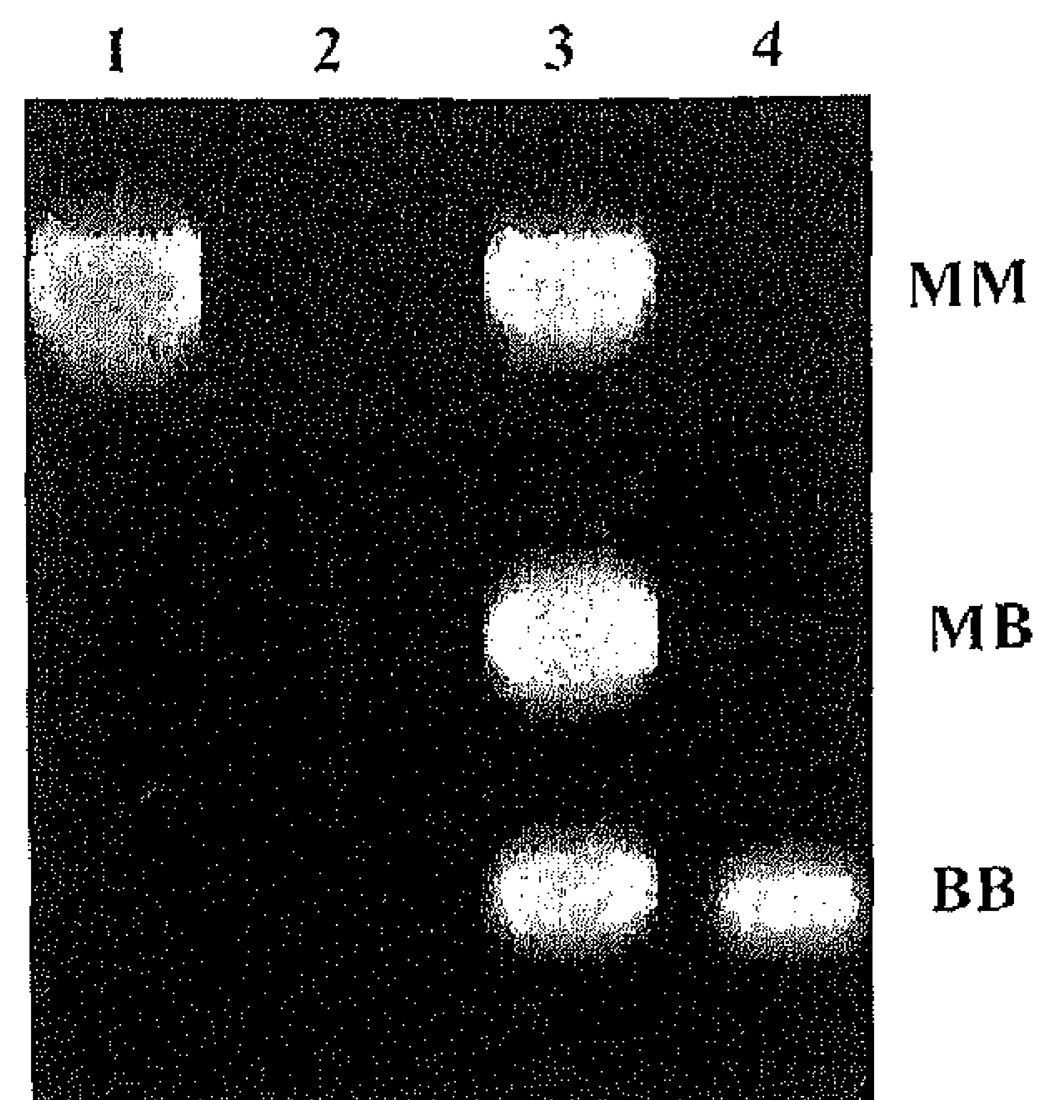


FIG. 1. **Creatine kinase isoenzyme gel of muscle homogenates.** Mating of M-CK KO (12) with mice overexpressing B-CK subunits (13) produced the switch mice with BB-CK replacing MM-CK in skeletal muscle. *Lane 1*, control muscle expresses the MM homodimer of CK; *lane 2*, M-CK KO does not express any cytosolic CK; *lane 3*, 7001 transgenic muscle (13) expresses the MM and BB homodimers as well as the muscle-brain heterodimer (*MB*); *lane 4*, switch muscle expresses only the BB homodimer. All samples are a 1:2500 dilution except for the M-CK KO, which is 1:300 dilution.

RESULTS

Fig. 1 shows a CK isoform gel that demonstrates that in the switch mice the only cytosolic form of CK present is BB-CK. Control animals had a total CK activity of 7980 ± 280 μmol min^{-1} g wet weight^{-1} (n = 6), and the M-CK KO had an activity of 350 ± 80 μmol min^{-1} g wet weight^{-1} (n = 6) due to mitochondrial CK that is still present in these muscles. Switch animals had 37% control activity with 2650 ± 600 μmol min^{-1} g wet weight^{-1} (n = 6) (Table I).

To measure the *in vivo* CK activity in the different mice ^{31}P NMR saturation transfer techniques were used (Fig. 2*A*). The decrease in the PCr peak during varying times with saturation of the γ-ATP peak was used to measure pseudo-first order rate constants in the different mice (Fig. 2*B*). The amount of PCr as determined from ^{31}P NMR was not significantly different among the different mice (data not shown), indicating that the rate constants can be used for a valid comparison of *in vivo* rates of CK. Pseudo-first order rate constants obtained for CK in control mice were 0.24 ± 0.02 s^{-1}. M-CK KO mice had a significantly lower value of 0.05 ± 0.05 s^{-1}. Switch mice had a rate constant of 0.14 ± 0.03 s^{-1}, which was 58% of control activity.

To determine if BB-CK localized to myofibrils in the switch muscle, immunofluorescence images of skeletal muscle sections stained with CK polyclonal antibodies were obtained (Fig. 3*A*). 4-μm tissue sections were immunoassayed, and stripes of specific staining on the sarcomeres of control muscle could be readily observed (Fig. 3*A*, *panel 1*). The M-CK KO mice showed no specific staining of the myofibrils (Fig. 3*A*, *panel 2*). There was also no specific sarcomeric staining in the muscle from switch mice as well, indicating that BB-CK did not specifically associate with myofibrils in the switch mice (Fig. 3*A*, *panel 3*). To further substantiate the lack of localization of BB-CK to myofibrils, CK activity was measured by isoenzyme gel electrophoresis from isolated washed myofibrils. Myofibrils from control mice contained significant amounts of MM-CK; however, no detectable CK was obtained from washed myofibrils isolated from switch mice (Fig. 3*B*).

To determine if BB-CK could rescue the phenotypes previously described in M-CK KO mice, alterations in markers of glycolysis and oxidative phosphorylation were measured (Table II and Ref. 38). As compared with control, M-CK KO mice had

TABLE I
Total in vitro CK activity at 37 °C obtained from superficial gastrocnemius muscle homogenates from control, M-CK KO, and switch mice

The results are reported as means ± S.D., $n = 6$.

	In vitro activity
	(μmol min^{-1} g wet weight^{-1})
Control	7980 ± 280
M-CK KO	350 ± 80
Switch	2650 ± 600

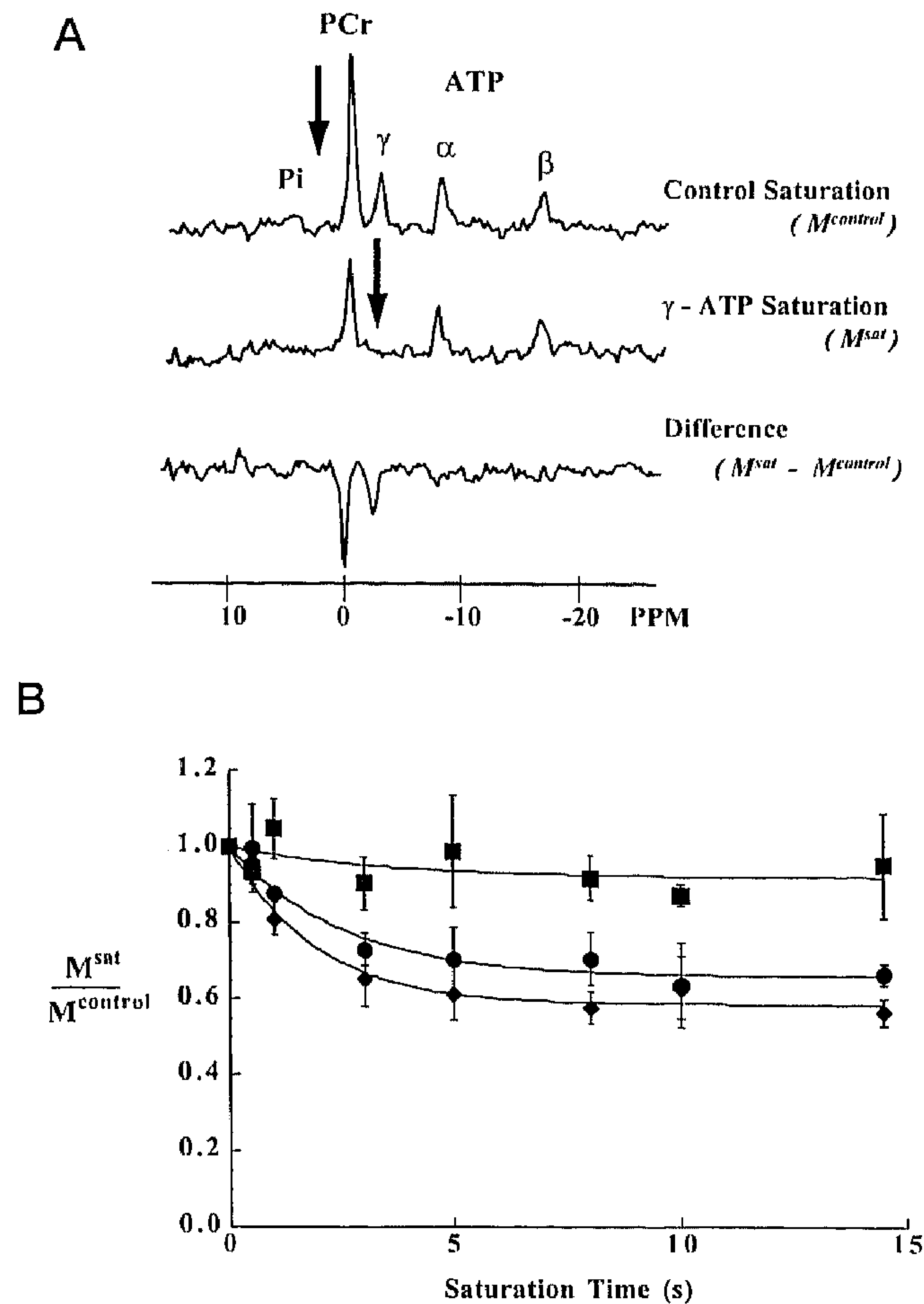
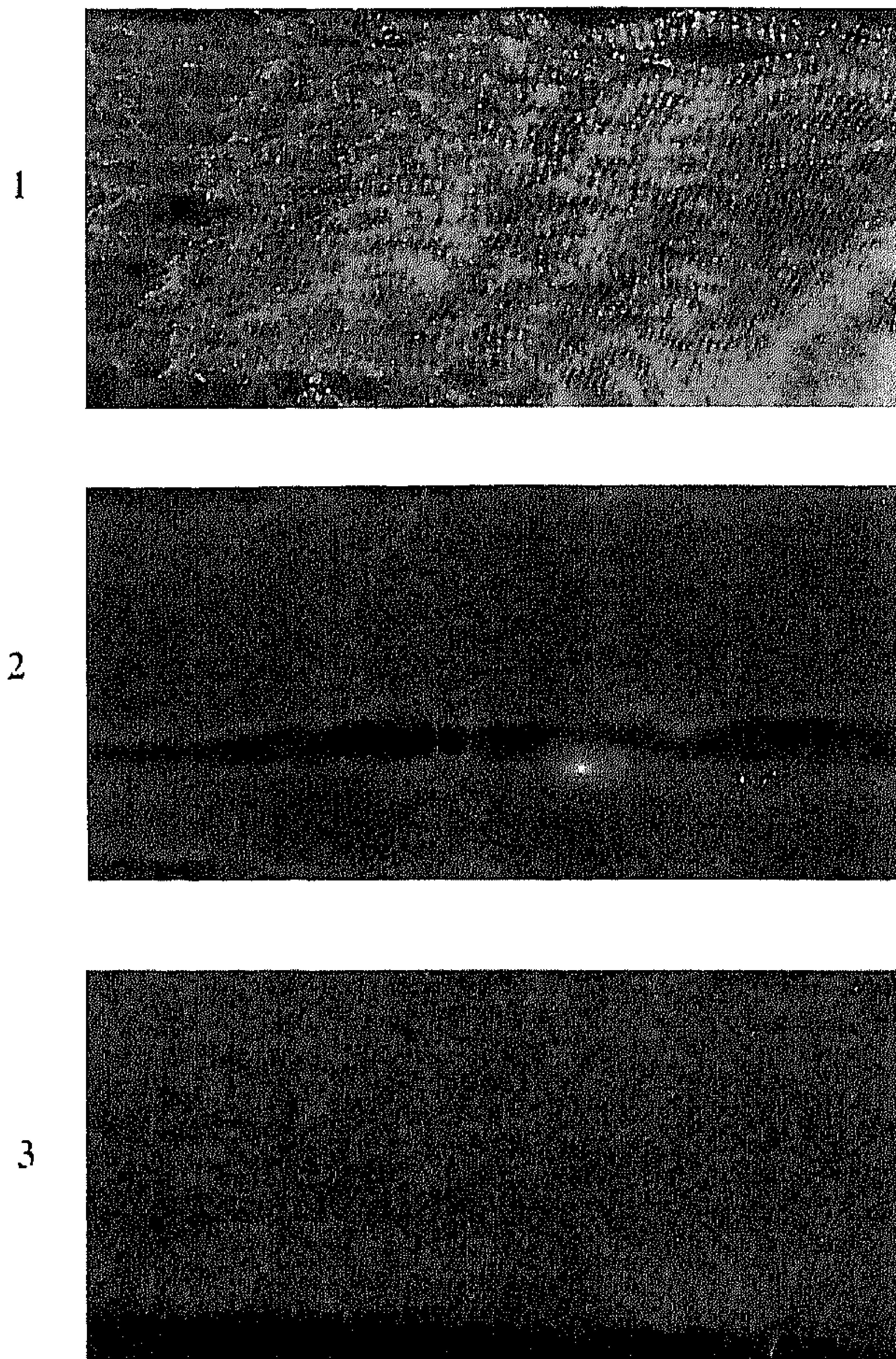


FIG. 2. **^{31}P NMR saturation transfer experiment to measure *in vivo* CK kinetics in skeletal muscle.** *A*, *in vivo* spectra from a switch mouse (that contains only BB-CK) skeletal muscle. Saturating pulses were applied at the control saturation position ($M^{control}$) as well as to the γ-ATP peak saturation position (M^{sat}). The difference between these spectra (difference $M^{sat} - M^{control}$) shows the decrease in PCr that is used to calculate the rate of the CK reaction *in vivo*. *B*, the time course of changes in PCr after saturating γ-ATP for control (♦), M-CK KO (■), and switch (●).



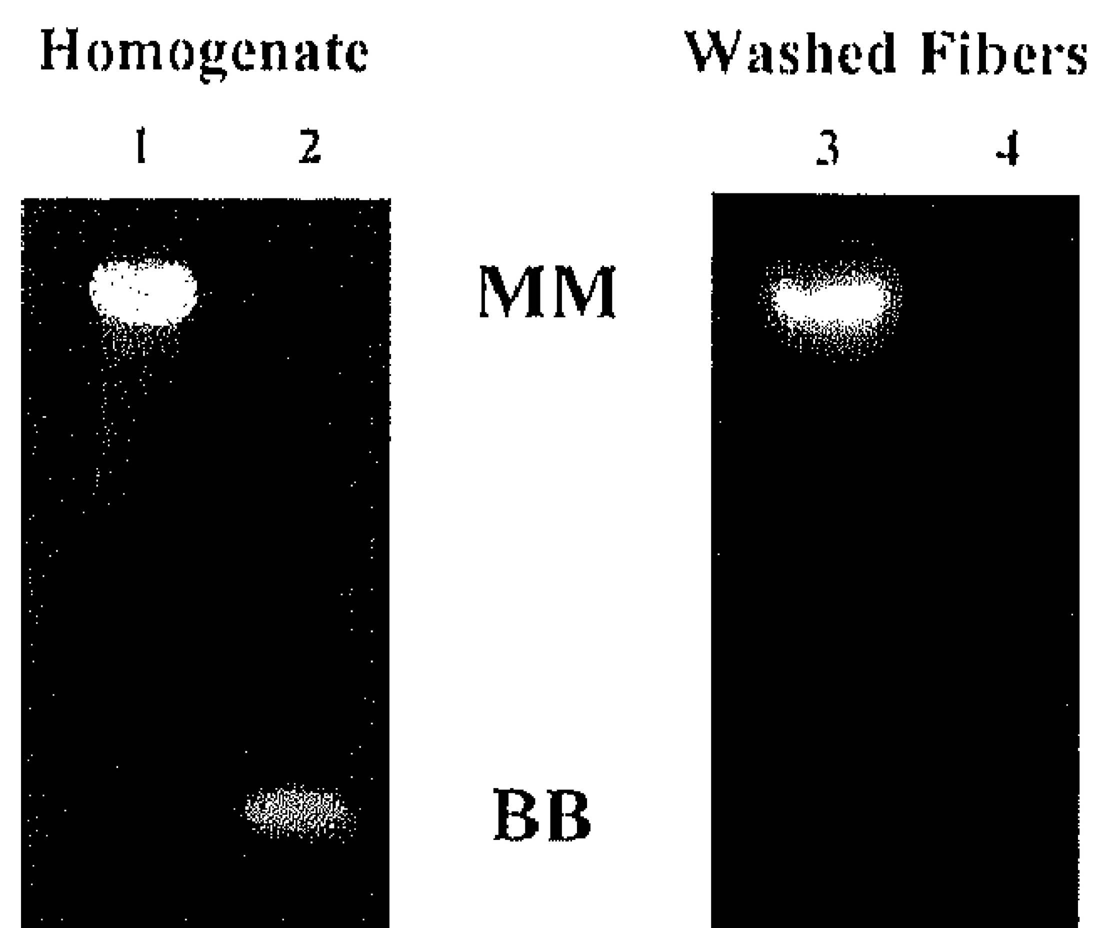


FIG. 3. **Localization of CK in muscle sections and isolated myofibrils.** *A*, immunoflourescence localization of CK to control (*panel 1*), M-CK KO (*panel 2*), and switch muscles (*panel 3*) (15). *B*, CK isoenzyme gels from isolated myofibrils. Homogenate containing myofibrils before (*lane 1*, control; *lane 2*, switch) and after (*lane 3*, control; *lane 4*, switch) washing three times (9).

significantly higher levels of glycogen and citrate synthase as previously reported (33). In addition, M-CK KO mice had significantly elevated levels of lactate dehydrogenate and adenylate kinase. In switch mice, the replacement of MM-CK with BB-CK led to a return to control levels of glycogen and lactate dehydrogenase. Citrate synthase levels were not significantly different from control in switch mice, although they tended to be intermediate between control and M-CK KO mice. The levels of adenylate kinase were elevated in the switch muscle compared with control and approximately equal to that of M-CK KO. Finally, no differences in myosin heavy chain isoforms were detected in control, M-CK KO, or switch mice (data not shown).

Fig. 4 illustrates examples of the mechanical records of force production from the different mice and time courses of changes in the average force generated as a function of contraction number. As previously reported, M-CK KO mice are not able to maintain force as compared with control early after initiating 5 Hz stimulation (33). M-CK KO mice only maintain 50% control levels of force after the 20th contraction. BB-CK rescued this contractile defect as illustrated by the fact that switch mice are able to maintain force as well as control mice (Fig. 4). No significant differences between switch mice and control were detected with a 5-s tetanic stimulation protocol as well (data not shown).

TABLE II
Biochemical assays from control, M-CK KO, and switch mice
The activities were determined at 37 °C in μmol/min/g wet weight. All results are means ± S.D., n = 6.

	Control	M-CK KO	Switch
Glycogen (mg/dl)	38 ± 6	67 ± 8	33 ± 8
Citrate synthase	19 ± 22	56 ± 20	41 ± 20
Lactate dehydrogenase	450 ± 60	580 ± 60	420 ± 40
Adenylate kinase	1260 ± 140	1700 ± 350	2000 ± 350

DISCUSSION

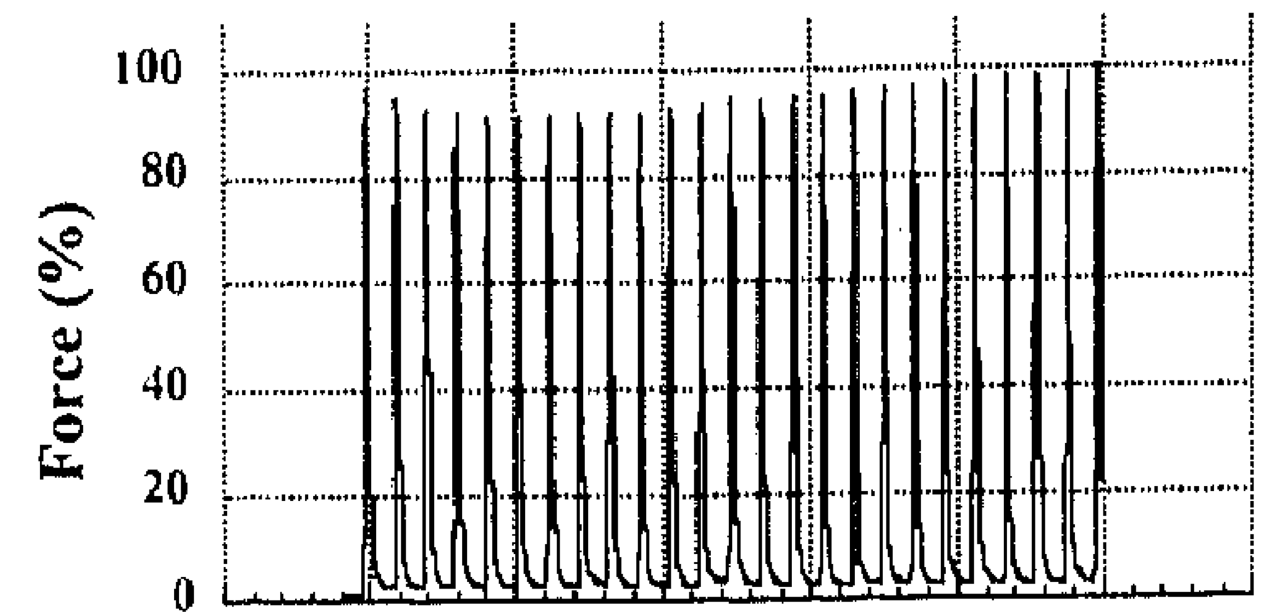
CK is central to cellular energy metabolism and is one of the best studied examples of an enzyme that exhibits subcellular compartmentation (13–16). Theoretical explanations for subcellular compartmentation of enzymes ascribe its importance to roles in metabolic control, enzymatic activity, and substrate utilization (10–12). These theoretical descriptions are supported by empirical experimental evidence showing that subcellular compartmentation occurs in multiple systems from metabolic enzymes (1–3) to tRNA channeling (7–9). To test the role of myofibrilar localization of CK, the consequences of a complete CK isoform switch from MM-CK to the BB-CK isoform, which does not localize to the M line (28, 29), were studied in transgenic mouse skeletal muscle. In MM-CK knockout mice there are large contractile defects (33–35). When MM-CK was replaced by BB-CK, the aberrant contractile phenotypes seen in MM-CK knockout mice were returned to normal despite the lack of myofibrillar localization. These results indicate that CK compartmentation to the myofibril of skeletal muscle is not essential for contractile function and that there is functional equivalence of creatine kinase isoforms in supporting cellular energy metabolism.

The activities determined from extracts represent V_{max} activities. It has been previously shown that the *in vivo* activity of CK is much less than the V_{max} activity in muscle (37). Previous results with different M-CK KO alleles indicated that the contractile defects and adaptation detected due to the lack of MM-CK were not measurable until CK V_{max} activities dropped below 20% of control levels and *in vivo* rates of MM-CK dropped below 30% (34). It is necessary to have BB-CK expression in the switch mice at or above these levels to begin to dissociate the importance of total CK from specific isoform activity. Compared with control levels, CK activity of switch mice is 37% from *in vitro* assays and 58% from *in vivo* assays as determined by ^{31}P NMR. Subcellular localization of CK was determined by two different techniques, isolated myofibrils and immunofluorescence of tissue sections. Both techniques indicated that MM-CK was localized to myofibrils but that BB-CK was absent from myofibrils. The data of enzyme activity and subcellular localization demonstrate that in the switch muscle BB-CK is present and active both *in vitro* and *in vivo* and is not associated with the myofibrils.

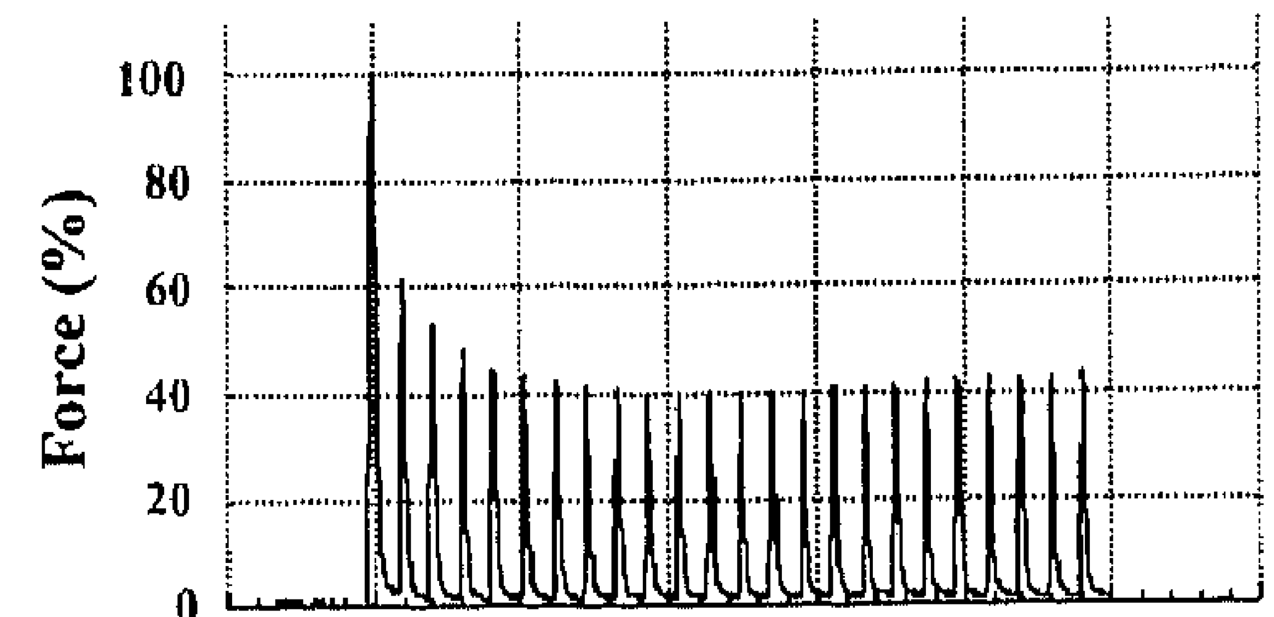
When CK levels are chronically modulated by chemical inhibitors or by genetic manipulation, there is usually an associated change in glycolytic and oxidative metabolites or enzymes (30–38). The complete set of metabolic adaptations seen in the M-CK KO mice (33) were not present in the switch mice. Most of these adaptations returned to the control state by the increased CK activity provided by BB-CK. Interestingly, adenylate kinase levels remained significantly elevated in the switch mice as compared with wild-type controls. Indeed, these results combined with other results on mice overexpressing the B-CK subunit in skeletal muscles (38) support the idea that adenylate kinase levels are adjusted in response to changes in CK levels; however, this may be independent of the isoform of CK present (39).

A

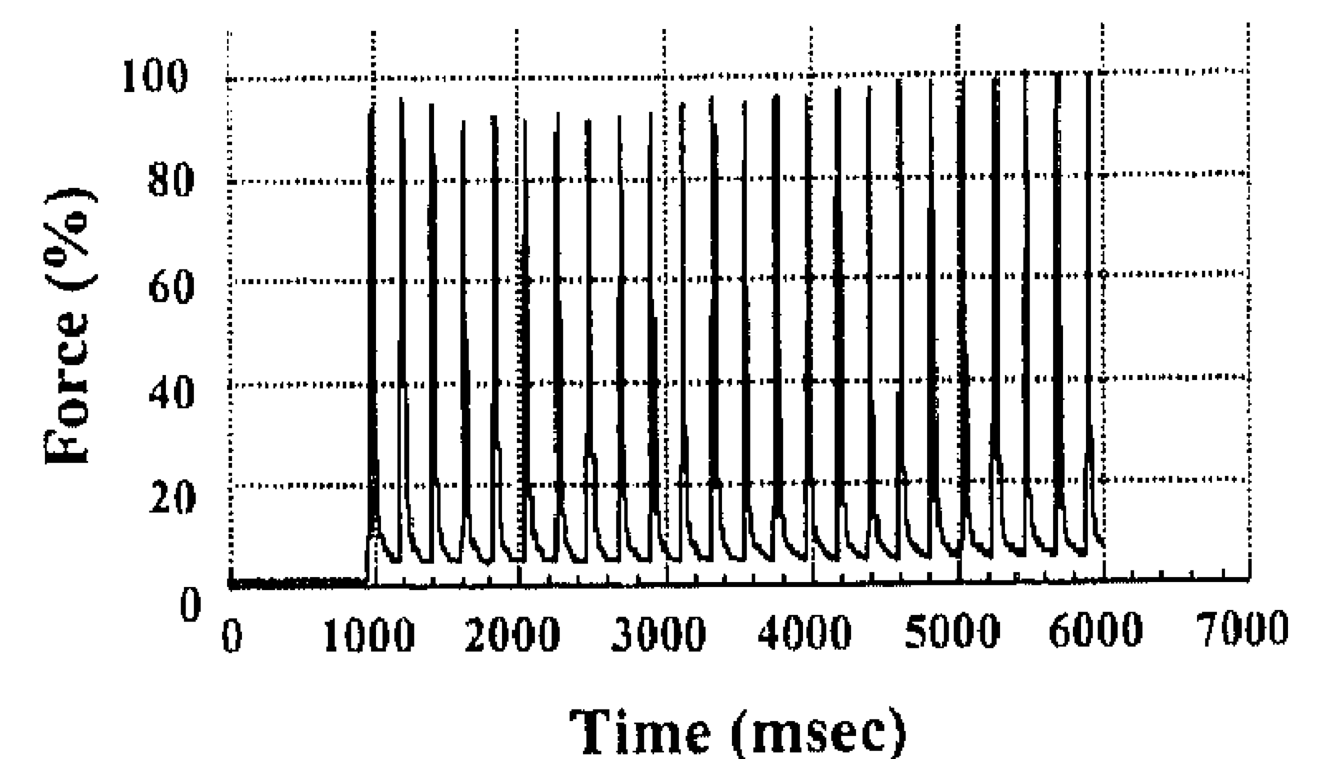
Control



M-CK KO



Switch



B

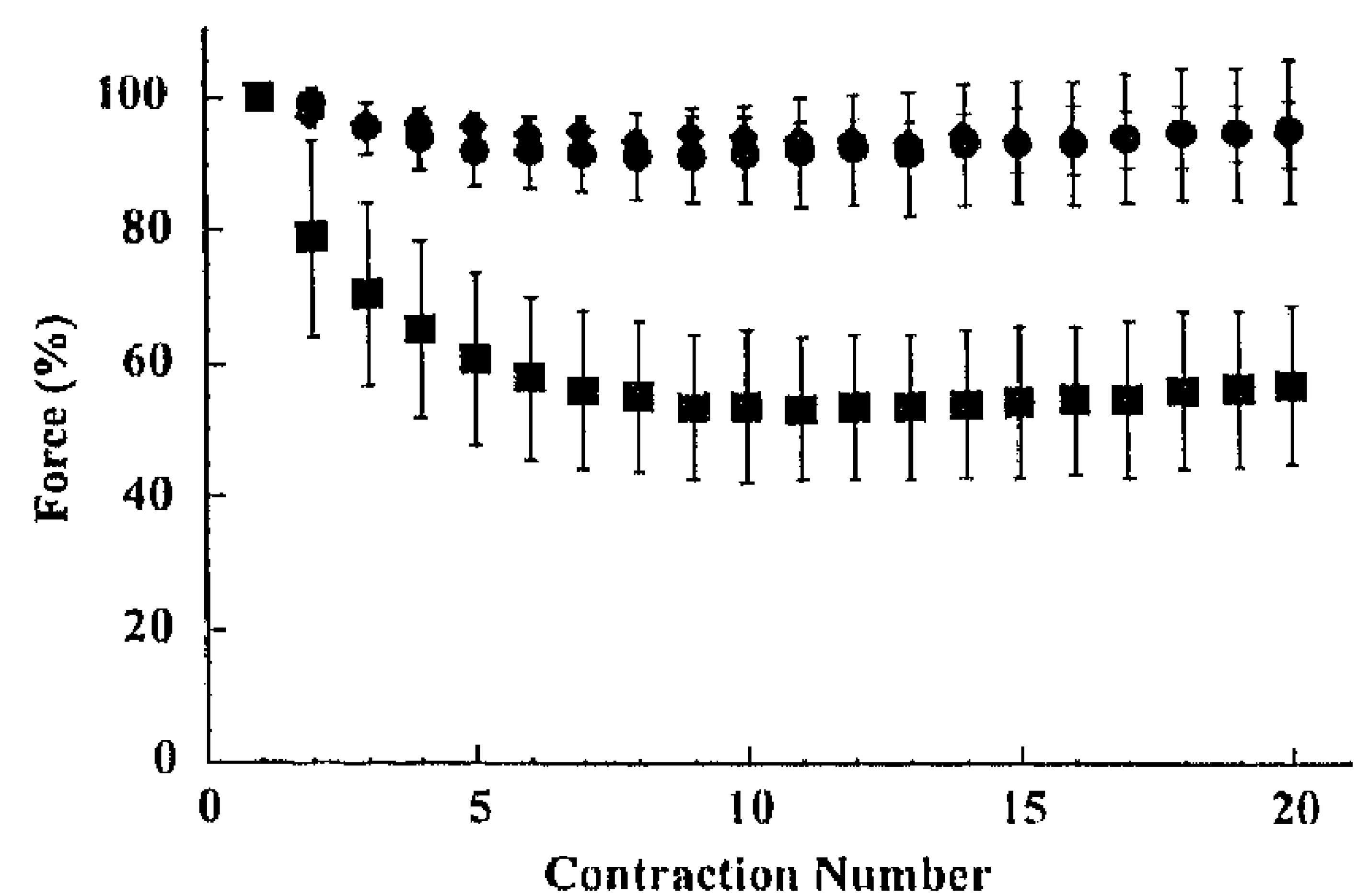


FIG. 4. **Contractile activity of control, M-CK KO, and switch gastrocnemius muscle.** *A*, representative 5 Hz isometric twitch force recordings as a function of time from the different muscles as labeled. *B*, graph showing average force production over the first 20 contractions from control (♦), M-CK KO (■), and switch mice (●). These results clearly illustrate the rescue of the contractile deficit in M-CK KO by the switch to BB-CK. The results are the means ± S.D. (n = 6).

The most important test to see if BB-CK could reverse the phenotypes detected in MM-CK KO mice was to measure force generation in the hind limb muscle. The stimulation protocol used resulted in muscles unable to maintain control levels of force generation, which is described as a lack of burst activity (33, 34). This mechanical adaptation to the lack of MM-CK clearly shows the importance of CK activity to muscle. Switch mice stimulated under identical conditions were able to produce control level force. Thus, the large decrease in force pro-

duction that occurs in mice lacking MM-CK was fully rescued by BB-CK present in the switch mice despite lower than control levels of CK and lack of localization to the myofibrils.

The phylogenetic tree of CK reveals gene duplication events that resulted in the multiple isoforms expressed today in cellular systems of all vertebrates and most invertebrates. Based on sequence homology there are distant evolutionary connections between CK and other guanidino kinases such as arginine kinase, the major guanidino kinase found in invertebrates (40). Conservation of guanidino kinases indicates an important role in cellular energetics for this class of enzyme. There is approximately 98% homology in MM-CK and 95% homology in BB-CK across species. The intraspecies homology between MM-CK and BB-CK is approximately 80%, which although less than interspecies homology within isoform is still extremely high (40). Interestingly there is sequence divergence between MM and BB-CK in the carboxyl-terminal of the enzyme. By expressing M-CK/B-CK fusions it has been shown that the carboxyl terminus of M-CK contains the information necessary for localization to myofibrils (41). In addition, differences in nontranslated regions of CK mRNA have been shown to cause localization of M-CK transcripts to myofibrils and control mRNA stability in B-CK transcripts (42). Thus, sophisticated systems for localization of both CK transcripts and protein have evolved.

The fact that there are divergent mechanisms that affect enzyme localization argues for potential differences in CK isoenzyme function. On the other hand, the large amount of homology indicates that MM-CK and BB-CK could play redundant roles in cellular energetics. Prior to the present study, the redundancy of CK isoforms in tissue function had not been tested. Results presented here demonstrate that BB-CK can rescue the contractile defects that occur in mice deficient in MM-CK. This rescue occurs despite the fact that BB-CK does not localize to myofibrils, indicating that localization of CK to the M line of myofibrils is not essential for force maintenance. This was true despite less than control levels of BB-CK. It may be that more subtle changes have to be examined to see a phenotype associated with lack of localization, for example, it may be that differences in MM-CK and BB-CK containing muscles may be revealed by comparing the effects of a more strenuous contractile protocol or long term training. These results support the idea that only a minimal level of CK is required for proper contractile function independent of localization. Initial results for heart and diaphragm from the M-CK KO mice indicate these tissues function like control, probably due to sufficient amounts of mitochondrial CK (43–45). Alternatively, it may be that localization of CK to other structures is important and that BB-CK is properly localized. For example, MM-CK has been shown to be associated with the sarcoplasmic reticulum, and recent evidence indicates that there are defects in calcium handling in M-CK KO and combined mitochondrial and M-CK KO mice (46). Finally, it is possible that metabolic adaptations substitute for any function of the localized forms of CK (43–45), although little evidence of adaptation was found in the switch muscle. Rather than serve a specific function, it may be that localization of metabolic enzymes to subcellular structures occurs to avoid macromolecular crowding in general in the cell (47). If true then disrupting the localization of any one enzyme will not have functional consequences. In conclusion, it is clear that for the protocols examined, BB-CK can replace MM-CK, despite lack of localization to the myofibrils.

Acknowledgments—We acknowledge technical assistance from Sean Alber, Eric Hoffman, Lisa Santel, Kathy Sharer, Ross D. Shonat, Afonso C. Silva, and Simon Watkins. We thank the Richard King Mellon Foundation, the Lucille Markey Charitable Trust, the Ralph M. Parsons Foundation, and the Ben Franklin Partnership Program of the Commonwealth of Pennsylvania for providing financial support for the establishment of the Pittsburgh NMR Center for Biomedical Research.

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