

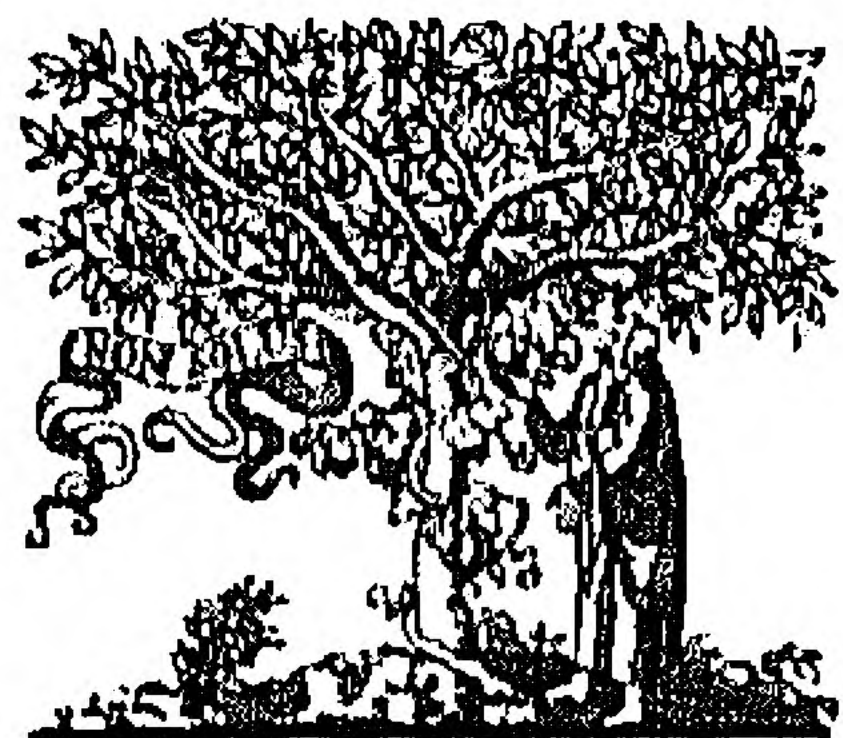
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# Mice deficient in ubiquitous mitochondrial creatine kinase are viable and fertile

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## Abstract

Creatine kinase isoenzymes (CK; EC 2.7.3.2) play a pivotal role in high-energy phosphoryl metabolism through subcellular compartmentation of the creatine-phosphate  $\rightleftharpoons$  ATP conversion reaction. In mouse, protein subunits constituting the ubiquitous mitochondrial CK (UbCKmit) and cytosolic B-CK isoforms are co-expressed in various cells and tissues with high and fluctuating energy demands such as brain, retina, smooth muscle, uterus, placenta and spermatozoa. Using targeted mutagenesis via homologous recombination in embryonic stem cells, we have generated mice that are deficient in UbCKmit subunits. These mice are viable and show no overt physical or behavioural abnormalities. Matings between UbCKmit-deficient mice produced normal numbers of offspring, showing that both females and males are completely fertile. Motility patterns of isolated spermatozoa were analyzed and found not to be impaired by absence of UbCKmit. From these results we conclude that UbCKmit is not essential for mouse viability, fertility, maintenance of pregnancy, or delivery.

*Keywords:* Creatine kinase; Mitochondrion; Gene targeting; Embryonic stem cell; Energy metabolism; Fertility

## 1. Introduction

The isoenzymes constituting the creatine kinase family are optimally suited to regulate high-energy phosphate homeostasis through functional coupling of CK subunits with subcellular sites of ATP production and consumption [1]. The individual members catalyze reversible phosphoryl-transfers from ATP to creatine (Cr), thereby generating phosphocreatine (PCr) and ADP via the reaction:  $\text{Cr} + \text{ATP} \rightleftharpoons \text{PCr} + \text{ADP} + \text{H}^+$ . Three cytosolic CK isoenzymes, CKMM, -MB, and -BB, are dimers composed of M (muscle) and/or B (brain) subunits and are encoded by two separate, but related, genes. Two additional nuclear genes encode distinct mitochondrial CKs, the ubiquitous (UbCKmit) and sarcomeric (ScCKmit) isoforms. CKmit appears in two interconvertible forms, octamers and dimers, of which the octameric molecule is thought to be the functional isoform in vivo. Cell-type specific co-expression of ScCKmit with M-CK in heart and skeletal muscle, and UbCKmit with B-CK in, for example, neurons, spermatozoa, photoreceptor cells of the retina, and smooth

muscle containing tissues like uterus, placenta and vessel walls (i.e., tissues with high energy demands) is generally accepted as strong evidence for a spatial energy buffering function of the CK/PCr system [for reviews see [2,3]].

The mitochondrial CK members are located along the outer surface of the inner mitochondrial membrane, and are enriched in energy-transfer contact sites between the two envelope membranes. Functional coupling of CKmit with tetrameric adenine nucleotide translocator (ANT) in the inner membrane and oligomeric voltage-dependent anion-selective channel (VDAC, porin) in the outer membrane drives the CKmit catalyzed reaction in the direction of PCr and ADP production, thus stimulating mitochondrial oxidative phosphorylation. Dynamic formation of these highly organized multi-enzyme complexes is enhanced upon increased mitochondrial activity and allows efficient channelling of high-energy phosphates, produced by oxidative phosphorylation, into the cytosol [3]. Within the cytoplasm, functional coupling of cytosolic CKs with ATPases and ATP-driven ion pumps increases the thermodynamic efficiency of these pumps because high local ATP/ADP ratios can be maintained [2]. Taken together, through subcellular compartmentation of the ATP/ADP-converting isoforms of the CK family, cellular energy

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transduction is mediated by a functional "PCr shuttle" with Cr and PCr as the metabolic intermediates [1], regardless of overall intracellular concentrations of ATP and ADP.

Expression of all CK isoenzymes is highly tissue-specific and developmentally regulated [4]. In the rat prenatal period, mitochondrial CK expression is only detectable in brain and intestine. After birth, upregulation of ScCKmit expression in striated muscle and UbCKmit in brain progresses in a time-dependent fashion which correlates with an increased mitochondrial mass and capacity for ATP synthesis by oxidative phosphorylation [4,5]. This implies a function of CKmit in coupling of ATP synthesis and demand in muscle tissue and neuronal cells. Increasing energy requirements, correlated with upregulated CK activities, are also associated with several phases of mammalian reproduction. Enhanced co-expression of B-CK and UbCKmit in uterus and placenta during rat pregnancy is regulated at transcriptional and translational levels [6]. The most convincing evidence for the importance of the CK/PCr system in high-energy phosphoryl transport comes from motility studies with sea-urchin spermatozoa [7]. Specific inhibition of CK activity leads to flagellar bend-

ing patterns that are indicative of insufficiencies in ATP delivery over longer distances.

The highly organized subcellular localization of CK isoenzymes and their substrates, combined with their functional association in multi-enzyme complexes, makes the CK/PCr system an ideal object for studying the role of metabolite channelling and compartmentalization in maintenance of energy homeostasis. Feeding of experimental animals with Cr analogues has been widely used to study effects of PCr deprivation in cardiac and skeletal muscle [8]. Similar studies on energy metabolism in brain have been hampered by the fact that these analogues have only little influence on the contents of Cr and PCr in brain [9]. Moreover, it is questionable whether these studies can also contribute to a better appraisal of the function of CKmit isoforms, for ScCKmit is not involved in phosphorylation of the Cr analogue  $\beta$ -guanidinopropionic acid in mouse skeletal muscle [10].

In an alternative approach we have chosen to elucidate the biological relevance of the CK/PCr system by studying effects of loss of function mutations in the CK family through gene targeting in mouse embryonic stem (ES) cells [11] and subsequent generation of CK-deficient mice.

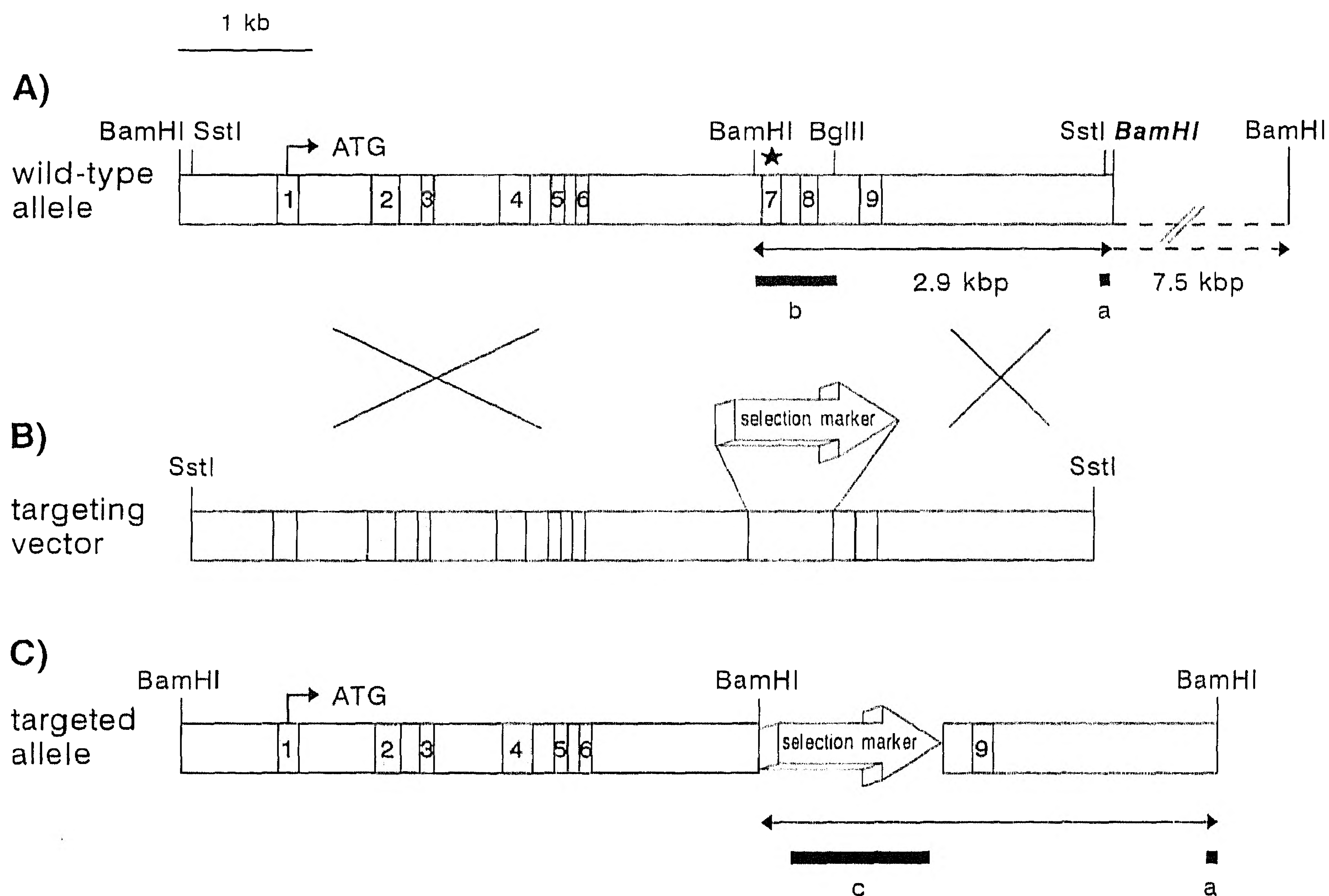


Fig. 1. Schematic diagram of the targeting strategy for disruption of the UbCKmit gene. Drawn are (A) structure of the wild-type UbCKmit gene locus in mouse strains 129/Sv and CBA or C57Bl/6, (B) the mouse strain CBA-derived targeting vector, and (C) the predicted structure of the UbCKmit locus after targeted mutagenesis in mouse 129/Sv embryonic stem cells. Numbers 1–9 denote exon sequences. Translation of the mouse UbCKmit gene is initiated in exon 1. The active cysteine in exon 7 is indicated by an asterisk. The arrow represents neomycin<sup>r</sup> or hygromycinB<sup>r</sup> selection cassettes. The arrowed lines indicate the diagnostic *Bam*HI fragments in wild-type and mutant alleles, as recognized by probes (a) a 100 bp *Sst*I–*Bam*HI fragment located outside the targeting construct, (b) the 600 bp *Bam*HI–*Bgl*II fragment that was deleted in the targeting vector and drawn as a striped box in figure (B), and (c) DNA fragments specific for the neo<sup>r</sup> or hygrob<sup>r</sup> genes. The polymorphic *Bam*HI site (written in bold italics) generates a 2.9 kbp fragment in wild-type mouse strains 129/Sv and CBA, and a 7.5 kbp band in the C57Bl/6 strain.



Earlier we have published that M-CK-deficient mice exhibit no overt abnormalities. Further study revealed, however, a crucial physiological role of the CK/PCr system in burst performance and a high degree of plasticity of cellular architecture and energy metabolism of muscle tissue [12,13]. As a first step in a similar study of the mitochondrial CKs, we have isolated the mouse UbCKmit gene and constructed a targeting vector thereof to generate mouse ES cells with both UbCKmit alleles mutated [14]. These cells revealed no abnormalities in their growing rate, differentiation potential, or rate of mitochondrial pyruvate oxidation. Complete absence of UbCKmit mRNA transcripts and hence also of UbCKmit activity was achieved by replacing the highly conserved region surrounding the catalytic cysteine residue with a neomycin resistance selection cassette. It was concluded that the constructed targeting vector would be suitable for generating UbCKmit-deficient mice. We now report on germline transmission of UbCKmit alleles carrying a similar type of mutation, and the subsequent generation of mice devoid of UbCKmit subunits. Consequences of deficiency of functional UbCKmit subunits on mouse viability and reproduction, and in particular on spermatogenesis, will be discussed.

## 2. Materials and methods

### 2.1. Construction of the targeting vector

A 7 kbp *Sst*I DNA fragment was isolated from a genomic lambda FIX<sup>TM</sup> II phage library of mouse F1-hybrid [CBA × C57Bl/6] spleen DNA (Stratagene, La Jolla, CA). The complete 4.8 kbp murine UbCKmit gene is encompassed within this genomic region (Fig. 1A). Starting from this subclone, a replacement-type vector for homologous recombination was constructed as described [14]. Shortly, from this DNA the 0.6 kbp *Bam*HI–*Bgl*II fragment, spanning exons 7 and 8 and coding for the pivotal cysteine, was deleted and replaced by either a 1.1 kbp neomycin resistance (*neo*<sup>r</sup>) cassette or a 2.0 kbp hygromycinB resistance (*hygro*B<sup>r</sup>) cassette (Fig. 1A and 1B).

### 2.2. Embryonic stem cell culture, electroporation and drug selection

Wild-type E14 ES cells [15] were a kind gift of Dr. Plump, Rockefeller University, New York, NY. Cells were grown on a layer of irradiated SNLH9 feeder cells [16] in Dulbecco's modified Eagle's medium supplemented with 15% (v/v) fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM β-mercaptoethanol. Prior to electroporation, ES cells were grown to subconfluency, trypsinized, washed two times and resuspended at  $1.8 \times 10^7$  cells per ml in culture medium. 20 μg of *Sst*I-digested targeting vector DNA was mixed with 0.8 ml cell suspen-

sion, introduced into the ES cells by electroporation at 500 μF and 250 V (Bio-Rad Gene Pulser) and plated on two 10-cm dishes containing irradiated feeder cells. Selection was applied 24 h after electroporation with 300 μg/ml G418 (Gibco) or 250 μg/ml hygromycinB (ICN Biomedicals, Aurora, Ohio, USA). Cells were allowed to grow for another 8–10 days before individual colonies were isolated and expanded for further analysis.

### 2.3. Southern blot analysis

Genomic DNA from individual ES cell clones or from tail biopsies of transgenic mice was prepared according to standard procedures [17]. For Southern analysis, approximately 8 μg of genomic DNA was digested with *Bam*HI, resolved by electrophoresis through 0.8% (w/v) agarose gels, and transferred to Biotrace nylon membranes (Gelman Sciences). DNA probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by the method of Feinberg and Vogelstein [18]. Probes for the UbCKmit gene were a 100 bp *Sst*I–*Bam*HI fragment located just 3' of the region used in the targeting vector (probe a), and the deleted *Bam*HI–*Bgl*II fragment (probe b), (Fig. 1A). For detection of the *neo*<sup>r</sup> and *hygro*B<sup>r</sup> genes, a 0.9 kbp *Bam*HI–*Eco*RI fragment from pMC1neo [11], and a 1.5 kbp *Pst*I probe from the *hygro*B cassette [19] were used, respectively. Hybridization was performed overnight at 65°C in 0.5 M sodium phosphate buffer (pH 7.4) containing 7% (w/v) SDS and 1 mM EDTA. Blots were washed to a final stringency of  $0.3 \times \text{SSC}/0.2\%$  SDS and exposed to Kodak X-Omat S1 film using intensifying screens.

### 2.4. Generation of UbCKmit-deficient mice

E14 clones which were diagnosed positive for homologous recombination and also contained the correct number of 40 chromosomes, were injected into recipient C57Bl/6 blastocysts and transferred into uterine horns of pseudo-pregnant (C57Bl/6 × CBA)F1 females [20]. Resulting chimaeric males were mated with C57Bl/6 females and germ-line transmission was scored by the agouti coat colour. Mice heterozygous for the mutant UbCKmit allele, as identified by Southern blot analysis, were intercrossed and null mutants were obtained. All examinations outlined in this paper were performed on F2-mice on a (C57Bl/6 × 129/Sv) hybrid background.

### 2.5. Creatine kinase isoenzyme electrophoresis

Freshly excised total brain tissue of mice at the age of seven weeks was homogenized with a teflon-glass Potter-Elvehjem homogenizer in a 1:10 dilution (w/v) of buffer containing 50 U/ml heparin, 250 mM sucrose, 2 mM EDTA, and 10 mM Tris-HCl at pH 7.4 (SETH-buffer) (4°C). For extraction of CKmit, homogenates were diluted 5-fold with 30 mM sodium-phosphate buffer (pH 7.4), 0.2



mM phenylmethanesulfonyl fluoride, 0.2 mM dithiothreitol, and 0.05% (v/v) Triton X-100 (Buffer A) [21]. Extracts were incubated at room temperature for 1 h and centrifuged for 30 min at 12 000 rpm and 4°C. Approximately 10 µg total protein of each sample was applied to a cellulose acetate membrane (Boskamp folien, LMB Laborservice, Bonn, Germany) and resolved by electrophoresis (200 V for 1 h) in Tris-barbital buffer (pH 8.6). CK activity was visualized with a colouring gel, prepared according to Kanemitsu and Okigaki [22] using a CK reaction kit (Boehringer, No. 1442376). Staining of adenylylate kinase (AK) activity was prevented by presence of 10 µM of the AK-specific inhibitor P<sup>1</sup>,P<sup>5</sup>-di(adenosine-5') pentaphosphate (Ap5A).

Spermatozoan CK was obtained by washing a spermatozoa preparation (see below) twice with PBS and resuspending the cells in 5 µl distilled water. Total protein was extracted by three rounds of liquid nitrogen freeze-thawing. One volume of buffer A was added, incubated for 1 h at room temperature, and centrifuged for 30 min at 12 000 rpm at 4°C. 11.5 µg of total protein of wild-type and mutant supernatants were loaded on a cellulose acetate membrane. As a reference, 1.4 µg of heart and 2 µg total brain extracts were applied. Proteins were separated and analyzed as described above.

#### 2.6. Western blot analysis

Freshly frozen tissues of total brain, skeletal muscle, testis and epididymis were homogenized at a 1:10 dilution (w/v) in SETH buffer at 4°C. Approximately 20 µg of total extracts were separated on 10% (w/v) SDS-polyacrylamide gels, and proteins were electrophoretically transferred onto nitrocellulose membranes according to Towbin et al. [23]. Non-specific binding sites on the blots were blocked overnight in 5% (w/v) non-fat dry milk in Tris-buffered saline (pH 7.6) with 0.1% (v/v) Tween-20 (TBST). CKmit protein was detected using subunit-specific polyclonal antibodies raised against synthetic peptides of

the UbCKmit or ScCKmit isoforms [24]. Antibodies were diluted 1:300 (αUbCKmit) or 1:5000 (αScCKmit) in TBST and incubated with the membrane for 30 min. The membranes were washed three times with TBST and incubated with goat-anti-rabbit immunoglobulin G coupled to horse raddish peroxidase (Amersham), diluted 1:10 000 in TBST, for 30 min. After three washes with TBST, immune complexes were detected by chemiluminescence (Boehringer) and exposed to Kodak X-Omat S1 autoradiography films.

#### 2.7. Spermatozoan motility measurements

Spermatozoa from wild-type and UbCKmit-deficient mice at the age of 12–16 weeks were isolated by disruption of the epididymi and vas deferens in 1–2 ml mouse tubal fluid medium (114.19 mM NaCl; 4.78 mM KCl; 1.19 mM KH<sub>2</sub>PO<sub>4</sub>; 1.71 mM CaCl<sub>2</sub>·2H<sub>2</sub>O; 1.19 mM MgSO<sub>4</sub>·7H<sub>2</sub>O; 5.00 mM NaHCO<sub>3</sub>; 4.79 mM sodium lactate; 0.37 mM sodium pyruvate; 3.40 mM glucose; 20.00 mM Hepes) [25], supplemented with 3% (w/v) bovine serum albumin (BSA), and kept at 37°C. Because motility of mouse spermatozoa decreases quickly in time, an effect which was enhanced by lowering the concentration of cells (personal observation), manipulations were kept to a minimum. Tissue debris was separated from the semen by a brief centrifugation step. Movement patterns of spermatozoa in the supernatant were measured in a µ-cell counting chamber (depth 20 µm; Fertility Technologies, Natick, MA, USA). Computer-assisted semen analysis was employed using the CellTrak/S system (Motion Analysis, Santa Rosa, CA, USA), mainly as described by Wetzels et al. [26]. Calibration of the CellTrak/S software used in this study was modified for use with mouse spermatozoa. Standard parameters calculated by the CellTrak/S system are percentage motile spermatozoa (MOT), straight line velocity (VSL), curvilinear velocity (VCL), mean linearity (LIN = VSL/VCL), and lateral head displacement (ALH).

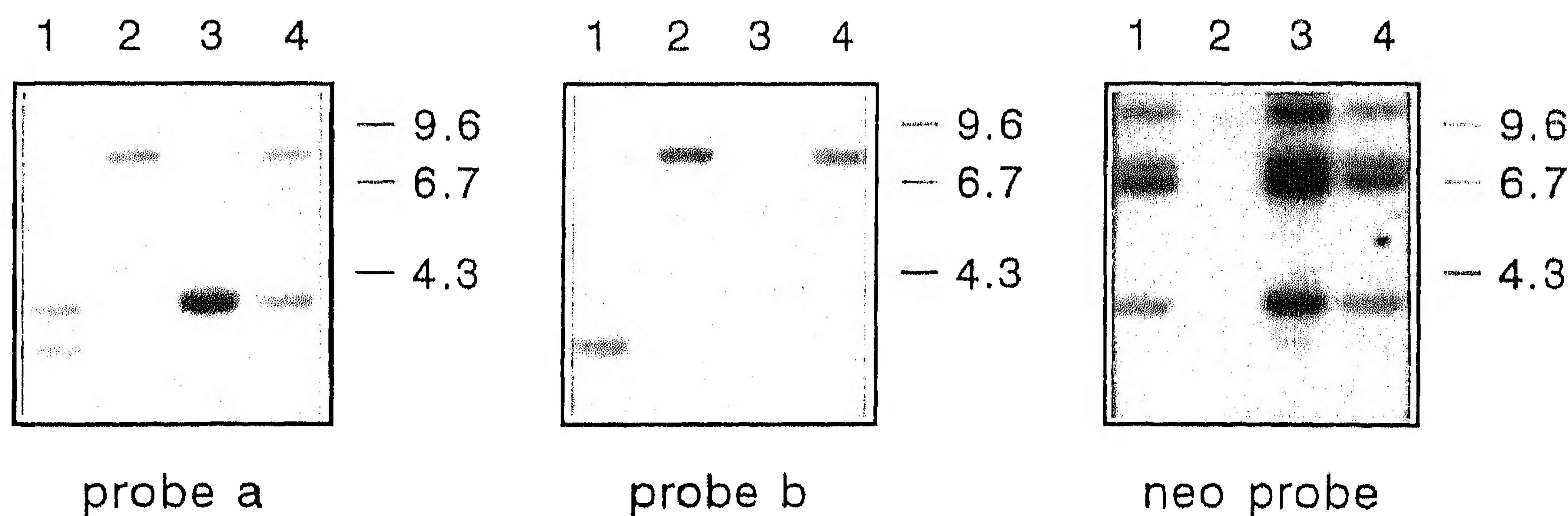


Fig. 2. Southern blot analysis of offspring from crosses between mice heterozygous for the targeted disruption of the UbCKmit gene. Genomic DNA was digested with *Bam*HI and subsequently hybridized with probes (a), (b) and (c; neo probe), as mentioned in the legend of Fig. 1. Lane 1 contains DNA from the original targeted E14 cell line with the neo<sup>r</sup> cassette replacing the *Bam*HI–*Bgl*II genomic fragment. In lanes 2–4, DNA from wild-type, homozygous mutant, and heterozygous littermates was loaded. Note the different hybridizing bands arising from wild-type 129/Sv (2.9 kbp) and C57Bl/6 (±7.5 kbp) *Bam*HI-digested DNA. Length of marker DNA fragment sizes is indicated in kbp at the right.



### 3. Results

#### 3.1. Disruption of the *UbCKmit* gene in mouse embryonic stem cells

We have previously reported on the cloning and characterization of the 4.8 kbp murine *UbCKmit* gene which is completely contained within a 7.0 kbp *SstI* fragment [14]. At the time of cloning the stimulation in targeting frequency in mouse ES cells by use of isogenic DNA constructs [16] was not yet recognized. Our genomic clone as depicted in Fig. 1A was therefore isolated from a library prepared from mouse F1-hybrid (C57Bl/6 × CBA) DNA. Genomic DNA comparison of mice of different inbred strains revealed a sequence polymorphism at the 3' proximal *BamHI* site, which is also the site used for identification of targeting events. A 2.9 kbp band is observed in mouse strain 129/Sv, the origin of the ES cells (see Fig. 2B, lane 1), while a fragment of approximately 7.5 kbp is detected in *BamHI*-digested C57Bl/6 DNA (see Fig. 2B, lane 2). This *BamHI* polymorphism assigns our genomic clone to be of CBA origin.

Targeting vectors were constructed in which *neo<sup>r</sup>* or *hygroB<sup>r</sup>* selection cassettes replace a 0.6 kbp DNA fragment spanning exons 7 and 8. This genomic sequence encodes the region containing the pivotal cysteine (Fig. 1). Stretches of homologous DNA sequences between targeting vector and the endogenous *UbCKmit* locus encompass 4.3 kbp at the 5', and 2.1 kbp at the 3' end, respectively. Wild-type E14 ES cells were transfected with *SstI*-digested targeting vector DNA and subsequently cultured in selective medium. Single resistant clones were expanded for DNA analysis by Southern blotting using diagnostic probe (a), located just 3' outside of the region used for the targeting vector. This probe recognizes a 2.9 kbp *BamHI* fragment in the wild-type situation, while introduction of *neo<sup>r</sup>* or *hygroB<sup>r</sup>* cassettes by homologous recombination results in hybridizing bands of 3.4 or 4.3 kbp, respectively. In two separate experiments, 18 out of 213 individually generated clones were found to be properly targeted. This is in agreement with previous experiments, showing high targeting frequencies of the *UbCKmit* locus, independent of the ES cell line or selection marker used [14]. Additionally, the cell lines were karyotyped to confirm the correct number of 40 chromosomes.

#### 3.2. Generation of mice deficient in functional *UbCKmit* subunits

Three independently derived E14 clones (one G418 resistant and two *hygroB* resistant), carrying a targeted *UbCKmit* allele, were injected into C57Bl/6 recipient blastocysts which were then reimplanted into pseudopregnant foster mothers. Highly chimaeric male mice, as judged by the proportion of agouti coat color, were obtained for all three cell lines. In a breeding program with C57Bl/6

females, only chimeras derived from the ES cell line with the *neo<sup>r</sup>* selection marker were able to transmit the ES cell derived genome to their offspring. Resulting animals that were heterozygous for the *UbCKmit* mutation were interbred to obtain mice with both *UbCKmit* alleles mutated. Litter sizes were normal and genotyping offspring by Southern blot hybridization with probe (a) showed the expected Mendelian distribution (Fig. 2A). Subsequent hybridization with the deleted 0.6 kbp *BamHI*–*BglII* fragment proved that homozygous mutant mice are true null mutants for the region surrounding the pivotal cysteine residue as no signal was observed (Fig. 2B). Unexpectedly, a complex pattern of bands appeared upon hybridization with the *neo*-specific probe (Fig. 2C). Most likely, a single replacement event occurred at the *UbCKmit* locus in the wild-type ES cell during which aberrant ligated vector sequences have integrated in the 5' region of the *UbCKmit* gene. Whether or not this is due to the use of homeologous vector constructs (CBA versus 129/Sv) is not known. Whatever, the region containing the essential cysteine residue at the 3' end was correctly replaced, as can be concluded from the signals with probes (a) and (b).

Since the region surrounding cysteine 278 is highly conserved in all CK isoenzymes, we anticipated deletion of this genomic region to prevent the gene from expressing functional *UbCKmit* subunits. To verify this expectation, cellulose acetate electrophoresis followed by a specific CK activity staining procedure was performed on total brain extracts of wild-type, heterozygous and homozygous mutant littermates (Fig. 3). Homozygous mutant mice are completely devoid of *UbCKmit* activity, while heterozygotes show approximately half of the level expressed in wild types. The two CKmit bands represent interconvert-

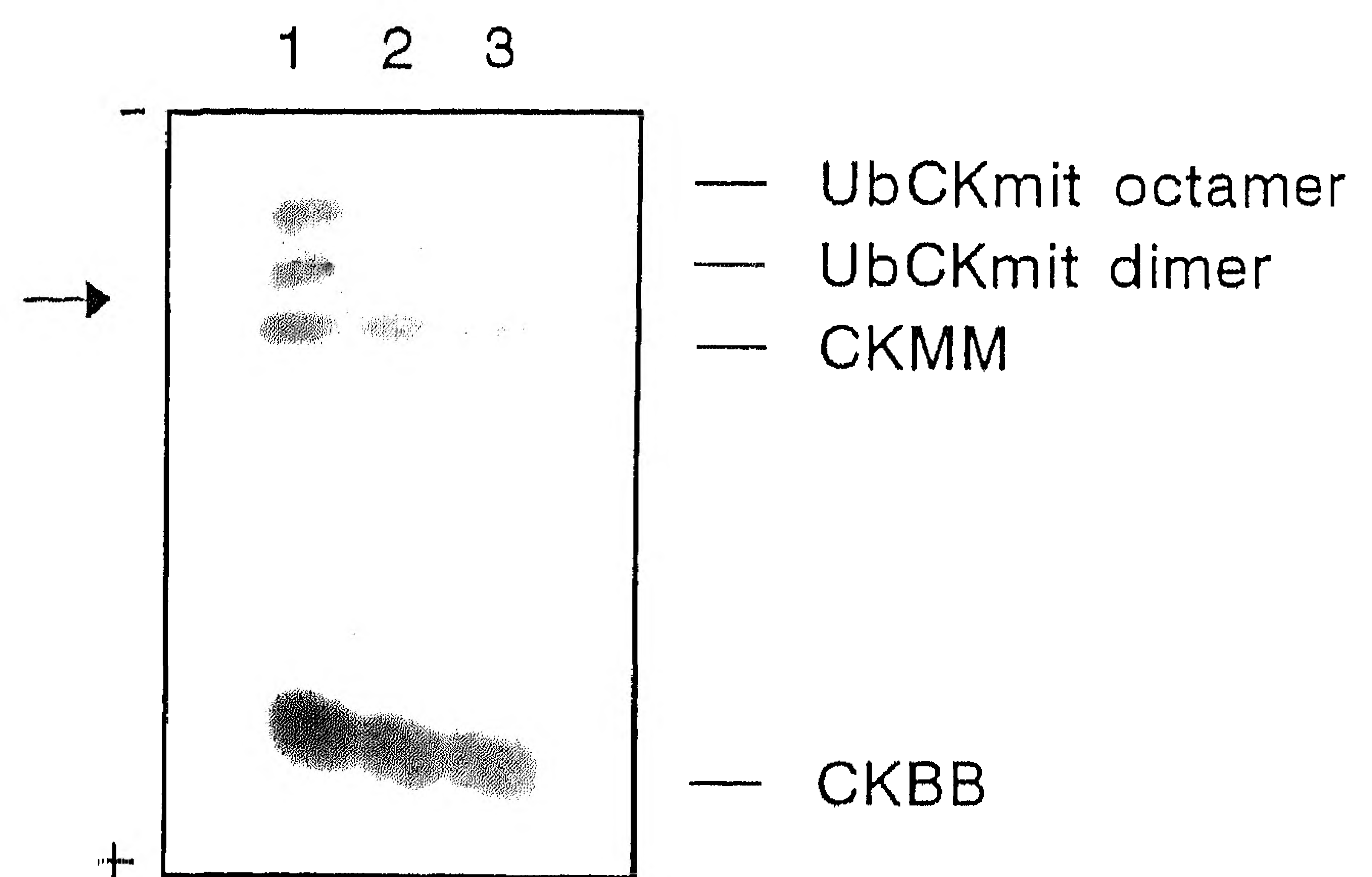


Fig. 3. Zymogram analysis of CK patterns in brain of offspring from mice heterozygous for the *UbCKmit* mutation. Extracts from total brain homogenates of wild-type (lane 1), heterozygous (lane 2), and homozygous *UbCKmit* mutant (lane 3) littermates were electrophoretically separated on cellulose-polyacetate membranes. Staining for CK activity was performed in the presence of the adenylate kinase-specific inhibitor Ap5A. Positions of CK subunit combinations found in brain are marked at the right. Loading position prior to electrophoresis is indicated by the arrow. Minus and plus mark the cathode and anode position, respectively.



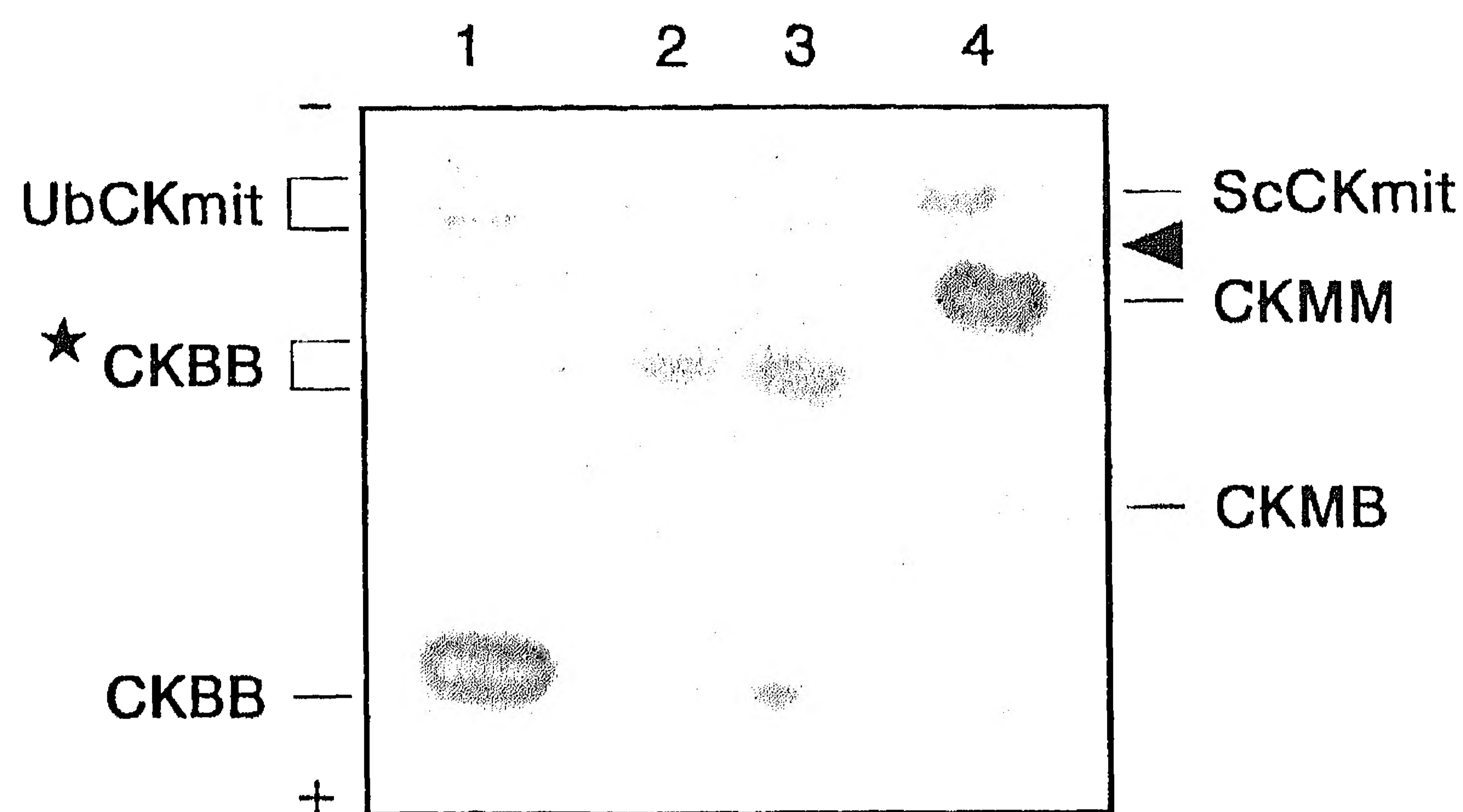


Fig. 4. Identification of the CK isoenzyme pattern in mice spermatozoa by zymogram analysis. Extracts of mouse wild-type brain (lane 1), UbCKmit-deficient spermatozoa (lane 2), wild-type spermatozoa (lane 3), and wild-type heart (lane 4) were electrophoresed under native conditions and stained for CK activity. Equal amounts of spermatozoan protein were applied to the membrane, while the amount of cardiac and brain protein was matched to allow simultaneous detection of all CK isoforms. Adenylate kinase staining was inhibited by addition of Ap5A. Positions of the various CK isoenzymes are depicted. Loading position prior to electrophoresis (arrowhead) and the orientation of cathode and anode are also marked. Mouse spermatozoa contain ubiquitous mitochondrial CK, as can be concluded from absence of this isoform from UbCKmit mutant mice. A relatively low amount of free CKBB is detected. The two extra bands, marked with an asterisk, which are specific for spermatozoa, could therefore represent associated or modified CKBB.

ible octameric (more cathodic) and dimeric (more anodic) CKmit molecules [27]. In extracts of UbCKmit-deficient brain no detectable compensatory expression of cytosolic CKBB or ScCKmit can be observed. Strikingly, a substantial expression of CKMM was detected in brain extracts (see also Fig. 4 for identification of CKMM with wild-type heart extract as a reference). Increasing the concentration of the adenylate kinase (AK) specific inhibitor Ap5A in the colouring gel from 10  $\mu$ M to 100  $\mu$ M had no effect on the resultant pattern of bands (result not shown). Therefore, the product in brain extracts which co-migrates with CKMM is not produced by remnant AK activity. Perhaps it should be noticed here that M-CK, and also ScCKmit, have been detected immunohistochemically in Purkinje cells of chicken [28] and also for rat brain there is evidence for localized presence of M-CK (B. Perryman, personal communication).

### 3.3. Normal reproduction of UbCKmit-deficient mice

Seven litters resulting from crosses between mice heterozygous for the UbCKmit mutation represented a total of 54 offspring. Genotyping the mice 2 weeks after birth resulted in identification of 12 wild types, 30 heterozygotes, and 12 homozygous UbCKmit mutants. From these numbers we can already speculate that carrying the mutated UbCKmit allele is not disadvantageous for either the developing oocyte or maturation and motility of spermatozoa.

To test whether UbCKmit-deficient mice are capable of normal reproduction, wild-type and mutant male and female animals were mated in the two reciprocal combinations. In addition, the mating behaviour and reproductive fitness of couples of UbCKmit-deficient males and females was tested. Litter sizes of UbCKmit-deficient females were not different from those of wild-type females, irrespective of the genotype of the males. Also, gestation times were not different for mutant mice compared to wild types. From these results we can conclude that active UbCKmit protein is not necessary for mouse spermatogenesis, oocyte growth, embryo development, support of pregnancy or the delivery process proper.

### 3.4. Characteristics of wild-type and UbCKmit-deficient spermatozoa

The identity of the CKmit isoenzyme in mouse spermatozoa was examined by zymogram analysis (Fig. 4). By comparison to total brain and cardiac muscle extracts, the

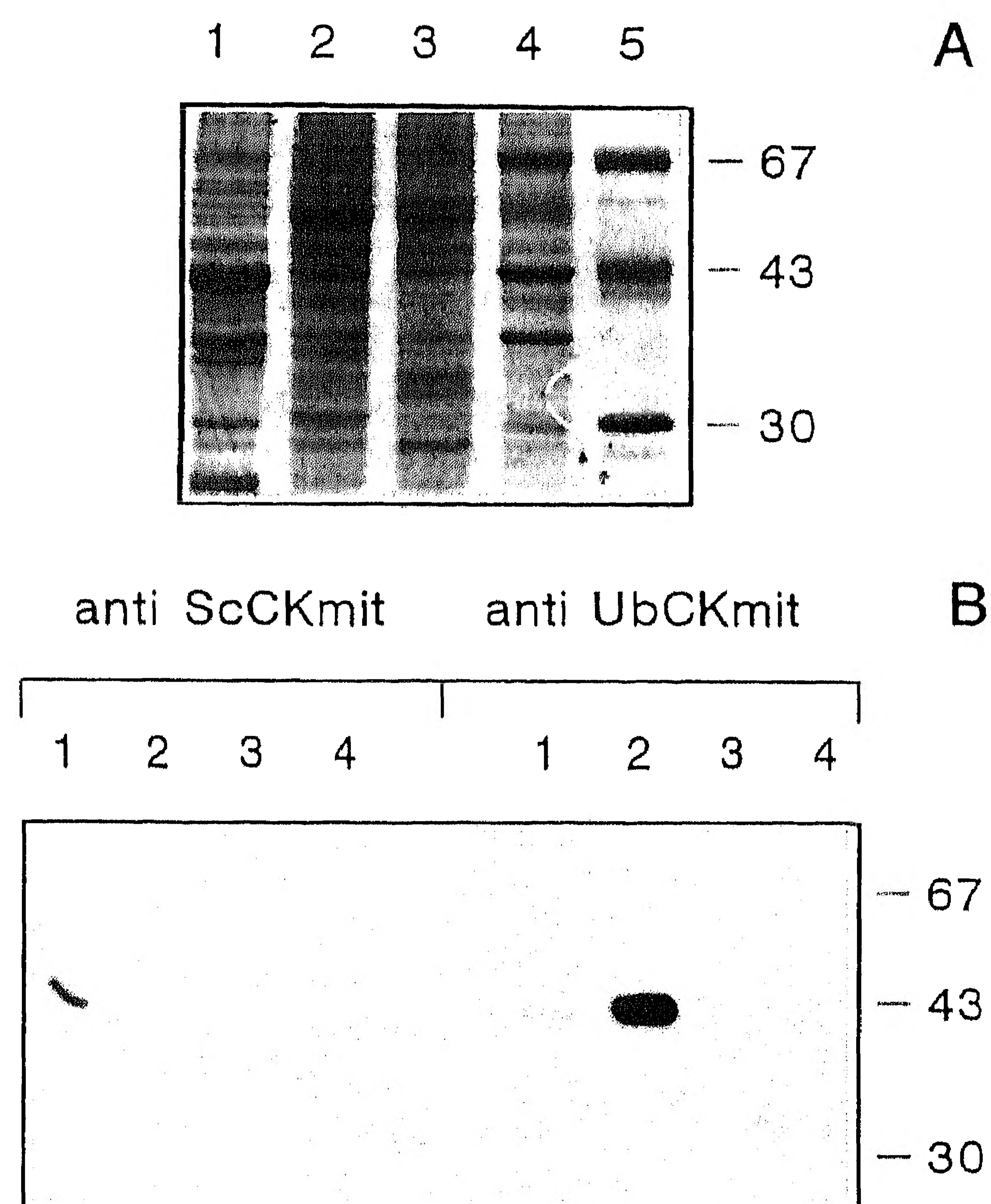


Fig. 5. Immunoblots showing subunit types of mitochondrial CK isoenzymes in the male reproductive system of mouse. (A) Protein bands of mouse skeletal muscle (lane 1), total brain (lane 2), testis (lane 3), and epididymis (lane 4) after SDS/PAGE electrophoresis and staining with Coomassie Brilliant Blue. Molecular mass markers were loaded in lane 5 and are indicated in kDa at the right. (B) Two identical gels with double protein amounts as in (A) were used for Western blotting. CKmit isoforms were identified using anti-ScCKmit (left) or anti-UbCKmit (right) specific antibodies and visualized by chemiluminescence. UbCKmit is expressed in mouse epididymis, but not in testis. ScCKmit is not detectable in the male reproductive system.



Table 1  
Motility analysis of isolated spermatozoa from wild-type and UbCKmit-deficient male mice

	Wild-type spermatozoa	UbCKmit-deficient spermatozoa
Motility	36.5 ± 11	26.5 ± 9
Straight line speed	23 ± 2	25 ± 4
Curvilinear velocity	76 ± 7	75 ± 6
Linearity	32 ± 3	36 ± 6
Lateral head displacement	6.2 ± 0.4	6.4 ± 0.7

Spermatozoa from wild-type and UbCKmit deficient males at the age of 12 to 16 weeks were isolated in MTF medium with 3% BSA. Spermatozoan motility patterns were recorded and analyzed using the CellTrak/S computer-assisted semen analysis system. The contribution of motile sperm (%), straight line speed (VSL;  $\mu\text{m}/\text{sec}$ ), curvilinear velocity (VCL;  $\mu\text{m}/\text{s}$ ), linearity (VSL/VCL), and lateral head displacement ( $\mu\text{m}$ ) were measured. Data represent mean  $\pm$  S.D. for six animals.

mitochondrial CK isoform in spermatozoa was identified as UbCKmit, and absence of this isoform in mutant males was confirmed. In contrast to mice, rooster spermatozoa express the muscle-specific form of mitochondrial CK [29]. This is remarkable since the cytosolic CK in spermatozoa of both species was identified as B-CK. The CKBB band is relatively faint in spermatozoan extracts of mouse (Fig. 4, lanes 2 and 3). Therefore, the two additional bands, which can only be detected in spermatozoa, could represent modified or protein-associated CKBB. For example, for sea urchin spermatozoa there is evidence suggesting that at least 10% of the flagellar CK might be membrane-associated [30]. Another fraction of cytosolic CK interacts with the microtubules of the axoneme, where it forms a functional compartment for rapid ATP-delivery to the dynein ATPase [31]. The experimental procedure followed by us might not have been stringent enough to dissociate all CKBB from these structures, but further study is necessary to explain the exact origin of the additional bands. Expression of CKmit isoenzymes in the male reproductive system was further analyzed by immunoblotting (Fig. 5). In mice, UbCKmit is expressed in epididymis, but is not detectable in testis. Antibody against a ScCKmit-derived peptide sequence does not generate detectable signals in either of these tissues. Taken together, these results provide strong evidence for UbCKmit being the single mitochondrial CK expressed in the reproductive system of the male mouse.

For examination of motility parameters, spermatozoa of wild-type and UbCKmit-deficient mice were isolated from the epididymis and vas deferens. Movement paths of spermatozoa were recorded and analyzed using the CellTrak/S computer-assisted semen analysis system. Although the contribution of motile spermatozoa was slightly lower in UbCKmit mutant males, no differences in linear speed, curvilinear velocity, linearity, or lateral movement of the head can be discerned between wild-type and UbCKmit-deficient spermatozoa, using this method of analysis (Table 1).

#### 4. Discussion

It is now generally accepted that cellular energy homeostasis is regulated via compartmentalized localization of adenine nucleotides and substrate-product channelling in multi-enzyme systems. One of these systems is the creatine kinase isoenzyme family, whose members are functionally coupled to sites of free energy production and consumption. Mitochondrial CKs are associated with the adenine nucleotide translocator in the outer mitochondrial compartment, and cytosolic CKs co-localize with myosin ATPase and several ion-transporting ATPases. Although extensive research has been devoted to the CK/PCr system, and very elegant model systems for its functioning have been proposed, its biological significance has remained an enigma.

We have used a previously reported targeting construct, carrying a neo<sup>r</sup> gene as the single positive selection marker, to generate mice with loss of function UbCKmit alleles. Deletion of the genomic region encoding the essential cysteine residue resulted in complete absence of UbCKmit activity in mutant brain. It is important to note that UbCKmit deficiency does not result in overexpression of striated muscle-expressed ScCKmit, nor in overexpression of its cytosolic counterpart, CKBB (Fig. 3). As a similar finding was observed for M-CK mutant mice [12], we may conclude that CK redundancy seems not to be involved in compensating mitochondrial or cytosolic CK deficiencies.

Mice homozygous for the UbCKmit mutation are viable and show no obvious phenotypic abnormalities in behavioural patterns, or responses to stimuli, like handling. This observation was unanticipated, particularly in view of the prominent role of the CK system in energy transfer reactions of brain which emerged from the first report on a patient who suffered from a severe extrapyramidal disorder caused by an inborn error of creatine biosynthesis [32]. Also *in vivo* <sup>31</sup>P-NMR experiments on adult rat demonstrated that there is a strong correlation between activity and the flux through the CK reaction in brain [33]. Several immunohistological studies revealed prominent presence of CK isoenzymes within various specialized cell types, with highest expression levels in Purkinje neurons [5,34,35]. Parallel expression of B-CK and UbCKmit in neuronal cells provides a structural basis for a functional PCr shuttle at these sites in the CNS [5,28,34].

The majority of ATP that is required to support neurotransmission and restore ionic gradients across neuronal membranes is generated by mitochondrial oxidative phosphorylation [36]. During postnatal mouse and rat brain development, the increased capacity for ATP synthesis by oxidative phosphorylation is accompanied by a 4–6-fold increase in CKmit expression, as well as appearance in the cerebellum of mitochondria with contact sites [5,37]. A variety of different experimental approaches have demonstrated functional coupling of octameric CKmit with oxidative phosphorylation [3]. Furthermore, defects in neuronal



bioenergetics are now increasingly being recognized as cause of progressive pathology in neurodegenerative diseases [38]. Therefore, a critical role for UbCKmit in mouse neuronal energy metabolism and motor learning capacity [39] was expected. Obviously, our findings did not meet this expectation. One likely possibility is that we did not sufficiently challenge functions of diverse brain centers in our animals to reveal effects from functional ablation of the mitochondrial compartment of the CK shuttle. Perhaps consequences of UbCKmit deficiency under standard housing conditions may only become relevant at later stages in adulthood, when it adds to the progressive decrease in mitochondrial electron transport with normal aging [40], and the neuron falls below a critical threshold in energy production. An additional, albeit less likely, explanation may be that loss of function at the most vulnerable sites is partly compensated by presence of low levels of the other CK family members, M-CK and ScCKmit, in the same cell types. Although presence of low levels of M-CK and ScCKmit has been reported for brains of chicken and rat ([28]; B. Perryman, personal communication), and although we could detect presence of fair levels of CKMM in our mouse brain preparations (Figs. 3 and 4), additional immunohistological distribution studies will be necessary to evaluate this possibility.

Functional credibility for a PCr shuttle mechanism in high-energy phosphoryl transport has been shown most convincingly by studies on the induction and inhibition of motility and its coupling to changes in PCr-pools of sea urchin spermatozoa [7,41,42]. Also there are experimental data that couple a low level of second CK isoenzyme, relative to CKBB, to a low fertilizing potential in man [43]. Furthermore, new ideas on aging, mitochondrial genetics and oxidative stress support the contention that low levels of ATP may be causally related to male infertility [44]. Spermatozoa are highly specialized polar cells that must be capable of delivering adequate amounts of ATP for the dynein ATPase at the distal end of the flagellum, while maintaining a sufficiently high ADP concentration at the mitochondria, located in the midpiece, to act as substrate for rapid, tightly coupled respiration [45]. The dependence of sperm motility on oxidative ATP production as well as ATP diffusion limitations may differ between species, but generally a functional CK/PCr shuttle is implicated.

Unfortunately, data on the exact isoenzyme patterns in spermatozoa from different species are still apparently conflicting. Bovine sperm have been reported to contain no creatine kinase at all and to depend largely on adenylate kinase activity for their energy supply [46]. CKBB expression in spermatozoa of rooster and humans has been identified by native isoenzyme electrophoresis and immunoblotting [47]. Surprisingly, the identity of the second CK enzyme was reported to be of the muscle specific ScCKmit type in rooster [29,47], and was cytosolic CKMM in humans [43]. Our isoenzyme distribution data point to

again a different situation in mouse spermatozoa. Native isoenzyme electrophoresis of extracts isolated from mouse spermatozoa confirmed presence of UbCKmit and CKBB for wild-type males, while absence of the former isoenzyme was evident in the mutants (Fig. 4, lanes 2 and 3). Furthermore, UbCKmit is expressed at detectable levels in mouse epididymis, but not in testis. This finding is in agreement with recent immunolocalization studies in mouse, using a monoclonal antibody against the cytosolic B-CK subunit [35]. ScCKmit protein could not be detected in either of these tissues (Fig. 5B).

Here we demonstrate that UbCKmit-deficient male mice are capable of normal reproduction, and that UbCKmit is not likely to be a key enzyme for mouse sperm function. Again within the margins of our test, detrimental effects of UbCKmit deficiency on motility patterns of mouse spermatozoa could not be detected. Therefore, we tentatively conclude that, in spite of the blockade in PCr transport from mitochondria to the cytosol, sufficient amounts of ATP are delivered to the dynein coupled ATPase to allow sliding of the microtubules. In contrast to sea urchin spermatozoa, vertebrate spermatozoa are able to thrive on both oxidative and glycolytic pathways for ATP production [48]. The possibility remains therefore that a shift in the amount of oxidative to glycolytically produced ATP, or maybe compensatory AK activity, provides a means of adaptation for our model.

Interestingly, CK action has also been suggested in other phases of mammalian reproduction, i.e. during the oocyte growing phase, and also following fertilization, until the eight-cell stage [49]. Further development of the embryo is accompanied by regulation of CK expression and activity in uterus and placenta during pregnancy. Hormonal control via estrogen responsive elements in the B-CK and UbCKmit genes is likely to be involved in transcription regulation during this period [6,14,50]. In rat and guinea pig, increased energy requirements of the parturient uterus are reflected in upregulation of levels or specific activity of UbCKmit together with an increase in oxidative capacity until immediately after delivery, while downregulation of placental UbCKmit before delivery may be a signal for the onset of placental senescence [6,51]. However, UbCKmit-deficient female mice, carrying UbCKmit-deficient embryos, are able to deliver and raise normal numbers of pups. Therefore, we cannot but conclude that functional UbCKmit is not crucial for normal mouse reproduction, embryogenesis, pregnancy and delivery.

Although we have thus far not been able to detect any overt phenotypic effects resulting from a loss of function mutation of the UbCKmit, the high evolutionary conservation that is observed for the CKmit genes, the strict regulation of transcription, and the expression of UbCKmit in a multitude of tissues and cells with discrete and specialized functions, do suggest a crucial function for this isoenzyme in metabolite communication between mito-



chondria and cytosol. Further detailed longitudinal examinations of our mutant animals should reveal whether the plasticity of cellular architecture and energy metabolism that was observed for M-CK-deficient muscles [12] also play a role here in protecting the phenotype.

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