Modulation of gene activity by consecutive gene targeting of one creatine kinase M allele in mouse embryonic stem cells

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ABSTRACT

The cytosolic creatine kinases (CK's; EC 2.7.3.2) BB, BM and MM are dimeric isoenzymes which have an important role in energy metabolism and display characteristic tissue- and stage-specific patterns of expression in mammals. To study the functional role of the distribution of the CK isoenzymes we have focussed on the modulation of expression of the genes encoding the individual B and M subunits, starting at the muscle creatine kinase (CKM) gene which is transcriptionally inactive during early embryogenesis. Using repeated rounds of gene targeting in mouse embryonic stem (ES) cells, two types of mutant cell lines were obtained. First, we generated a cell line in which insertion of a neomycin resistance (neo^r) gene had disrupted one of the CKM alleles. Subsequently, from this cell line, following introduction of an insertion type vector designed for replacement of the muscle specific CKM-enhancer by the constitutively acting polyoma virus enhancer PyF441, several independent doubly targeted clones were isolated which all had insertions in the previously neo-disrupted CKM allele. In some of these ES clones, the targeted enhancer replacement resulted in gene correction and functional activation of the silent CKM gene. Dimerisation between the ectopically expressed CKM subunits and CKB subunits which are normally present at high levels in ES cells, led to the formation of the BM isoform of CK in these clones.

INTRODUCTION

Creatine kinases play a pivotal role in the energy metabolism of mammalian cells by maintaining appropriate levels of intracellular ATP/ADP and creatine phosphate. The CK-enzymes function by catalysing the end-reactions of the high energy phosphate shuttle involved in transport of energy from mitochondria to cytosol (1). In mammals, three dimeric cytoplasmic CK isoforms, CK-BB, -MB and -MM are known to exist (2, 3). These CK-variants can partition reversibly between kinetically distinct soluble and protein or membrane-bound forms, with the distribution influenced by energy demand and tissue type (1, 4). In conjunction with the cytosolic CK's two mitochondrial isoforms (Mi-CK's), associated with the exterior surface of the inner mitochondrial membrane, are found (5, 6).

The two distinct B- (for Brain) and M- (for Muscle) subunits of the cytosolic CK-dimer isoforms are encoded by two different, though strongly related, genes (CKB and CKM) which are positioned at different chromosomal locations in the mammalian genome (7–9). The expression of both genes is tissue-specific and developmentally controlled by complex regulatory mechanisms which lead to characteristic tissue- and stage-specific isoform patterns (10, 11).

In order to obtain a better understanding of the biological role of the cytosolic CK distribution patterns in vivo, we have decided to establish cellular and animal models that will enable us to modulate the expression of the individual isoenzyme genes in a controled fashion. Ideally, studies into the role of isoenzyme distributions require genetic backgrounds that encode null alleles of the genes of interest. Gene targeting by homologous recombination in embryonic stem cells recently has been established as one of the most powerful genetic methods for gene ablation and mutagenesis with accurate control over gene copy number and transgene position effects (12–16). We therefore descided to use this technology for changing the expression of the CK isogenes, starting with the mutagenesis of the CKM gene. The CKM gene is transcriptionally activated relatively late in embryonic development during the process of terminal differentiation of myoblasts, in which trans-acting factors like MyoD are involved (17). Well characterized cis-regulatory enhancer elements are situated around position −1100 and within a 900 nucleotide (nt) region of the first intron (E1 and E2, respectively; 18–20). In addition, an AT-rich regulatory segment common to both CKM and CKB genes and located just downstream of the E1 enhancer, has been identified (21).

We report here on a gene targeting procedure, that enabled us to develop ES cells with loss or gain of function mutations

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in the CKM gene in a consecutive fashion. First, gene inactivation at one CKM allele was achieved by the introduction of a neo^r cassette which interrupts the body of the gene and replaces the segment spanning the translational start site. Subsequently, from this mutant CKM gene, a new constitutively producing variant was derived by introducing an insertion vector which exchanges the −1100 enhancer region for the polyoma virus enhancer and shifts the normal regulatory elements to a position approximately 19 kb further upstream. The relevance of some conspicuous observations in our targeting experiments are discussed.

MATERIALS AND METHODS

Preparation of targeting vector plasmids

To construct targeting vector pRV8.5, a 9.0 kilobase pairs (kbp) genomic BamHI fragment spanning exons 1−5 of the CKM gene and derived from a mouse CKM specific recombinant lambda phage that originated from a Balb/C genomic DNA library in phage EMBL3 (a gift of Drs. G.Grosveld and D.Meijer, Erasmus University, Rotterdam) was used as starting material. From this DNA, a 0.5 kbp HindIII fragment encompassing part of intron 1 and exon 2 was removed and replaced by a 1.2 kbp HindIII fragment containing a neo^r cassette. This cassette contained a 1.1 kbp XhoI/BamHI fragment carrying the neo^r gene from plasmid pMC1neo (22) which, at its 3′ end, was provided with a 58 base pair (bp) Sau3A fragment that spans the polyadenylation signal of plasmid pMC1neoPOLA (Stratagene).

Insertion vector pIV9.0 was obtained by ligating a 2.0 kbp hygroB^r cassette to the 5′ end (at nucleotide position −808; (19)) of the 9.0 kbp BamHI CKM gene-fragment. The hygroB^r cassette consisted of a 1.8 kbp PvuII/BglII fragment of vector pECV6 (23) containing the thymidine kinase (tk) promoter, the entire coding sequence of the hygroB^r gene, the tk polyadenylation signal and, at the 3′ end, a 180 bp EcoRI fragment spanning the polyoma mutant enhancer region, EPyF441 (24), derived from plasmid pMC1Neo (22). All cloning manipulations were according to standard procedures (25).

Embryonic Stem cells

The wild-type (wt) male ES cell line ES5, derived from mouse strain 129 blastocysts (26) was obtained from Dr. C. Mummery, Hubrecht Laboratory, Utrecht. Mouse embryonic CCE stem cells (27) were obtained from Dr. M. Evans, Cambridge UK. Cells were continuously grown on gelatine coated culture flasks in 60% Buffalo rat liver (BRL)-conditioned Dulbecco's Modified Eagle's Medium (DMEM) (28) supplied with 20% fetal calf serum (FCS). Medium was changed daily and cultures were split every 3−5 days.

Electroporation and drug selection

Targeting vector DNA was introduced into ES cells by electroporation (TA750 transfection apparatus, Krüss GmbH Hamburg, FRG) (29). Batches of $2.5-3.0\times10^7$ ES cells were resuspended in 0.4 ml electroporation buffer [0.28 M sucrose, 1 mM K-phosphate (pH 7.1), 0.5 mM $CaCl_2$ and 0.1 mM MgCl2] mixed in with 25 μg/ml BamHI (pRV8.5) or EcoRV (pIV9.0) linearized vector DNA . The cells were given a single pulse (100 μs, 2.5 kV per cm), then allowed to stand at room temperature for 20 min before plating onto 9 cm diameter tissue culture petri dishes in 60% BRL-conditioned medium. Typically, the electroporation conditions resulted in 75−85% survival. Clonal selection of ES5 cells was started 24 hr later by adding medium containing 500 μg/ml of G418 (Gibco/BRL) for the first round of mutagenesis. For the second round of mutagenesis 200 μg/ml of hygromycin B (Boehringer Mannheim GmBH) was used. For selection of CCE cells, the drug concentrations were reduced to 250 μg/ml G418 or 100 μg/ml hygromycin B, respectively. The selective media were changed every 2−3 days. Ten to fourteen days after transfection individual colonies were picked and expanded for DNA preparation and storage.

Southern Blot analysis of genomic DNAs

Genomic DNAs were prepared from batches of approximately $1-2\times10^6$ ES cells originating from individual drug resistant clones. Aliquots of approximately 5 μg of DNA were digested with the appropriate restriction enzymes using conditions as specified by the manufacturer. DNA fragments were resolved on 0.8% w/v agarose gels, transferred to Biotrace Tm nylon membranes (Gelman Sciences Tm) and hybridized to ^{32}P-dCTP (Amersham) labeled probes. Probes for CKM, a 900 bp BamHI-EcoRI fragment spanning exon 6 and part of introns 5 and 6 (designated CKM-3′ probe in Figs. 1 and 2), the replaced 500 bp HindIII fragment spanning the ATG codon (designated CKM-ATG probe in Fig. 2), the neomycin gene, a 900 bp EcoRI-SalI fragment derived from pMC1neo (22) and the hygromycin gene, a 331 bp PstI fragment derived from pECV6 (23), were prepared by isolating the appropriate fragments of restriction enzyme digested plasmid DNAs after electrophoresis in low-melting agarose (Bio-rad). The DNA fragments were labeled by the random priming procedure of Feinberg and Vogelstein (30) using hexanucleotide primers (Pharmacia LKB Biotechnology) with Klenow DNA polymerase in the presence of [α-^{32}P]dCTP (Amersham International UK) and the three unlabeled triphosphates. Blots were hybridized in 0.5 M NaPO4 buffer containing 7% w/v SDS and washed at a final concentration of 40 mM NaPO4, 0.1% SDS at 65°C. Exposure to Kodak X-O mat film was for 1 to 3 days at −70°C.

Zymogram typing of CK isoenzymes

Typing of cytosolic CK isoenzymes in targeted ES cells and neuro-muscular tissue extracts was done by electrophoresis followed by CK activity staining. Briefly, 1×10^7 ES cells, were suspended in 250 μl CK-NAC buffer (Boehringer Mannheim GmBH) containing 1 mM Phenylmethylsulfonylfluorid (PMSF), lysed by three rounds of freeze-thawing and centrifuged at 13000g for 2 min. Two μl cell lysate or mouse tissue homogenate was applied to a cellulose acetate membrane (Sartorius) and resolved by electrophoresis (300 V for 30 min) in Tris-barbital buffer pH8.6 (31). The positions of CK activity were vizualized by activity staining according to Kanemitsu et al. (32).

RESULTS

Disruption of the CKM gene in ES cells

As a first step, a replacement-type targeting vector, pRV8.5, was designed to functionally inactivate the mouse CKM gene (22). In pRV8.5 DNA, a CKM region of approximately 0.5 kbp encompassing part of intron 1 and exon 2 and including the ATG translational start codon, was replaced by a 1.2 kbp fragment carrying the neo^r gene (Figure **1A**). BamHI digested pRV8.5 plasmid DNA was introduced into ES5 cells by electroporation, G418 resistant colonies were selected and clonally expanded, and genomic DNA was isolated to directly identify homologous recombinants by conventional Southern blotting. Individual

genomic DNAs were digested with EcoRI and analysed using a CKM specific probe located outside the introduced DNA fragment (probe CKM-3′ located at the 3′ flank of the 9.0 kbp gene segment ; fig. 1). With this probe, mutants were expected to yield both the wild-type 6.5 kbp fragment between the EcoRI sites in introns 1 and 6, as well as a novel 6.7 kbp fragment flanked by EcoRI sites inside the neo^r cassette and in intron 6. This mutant 6.7 kbp fragment was also expected to hybridize with the neo probe. Using these signals as a diagnostic tool, we identified one homologous recombinant (ES5-2697) out of 158 neo^r ES5 colonies screened (Table 1). For reasons unclear, the mutant 6.7 kbp EcoRI fragment consistently yielded a substoichiometric signal as compared to the wt CKM allele in our Southern analyses. Similar differences in allelic signals have been observed for other gene targeting experiments by others (16,33,35). To obtain further evidence for the anticipated integration in clone ES5-2697, we used a polymorphic XbaI site in the CKM gene (see Fig. 1) which is present only in Balb/c derived targeting vector DNA but absent in ES5 DNA originating from mouse strain 129. As this site is relatively close to the neo insert it is especially well suited to monitor the fate of the incoming DNA. Upon probing XbaI digests with the CKM-3′ probe we expected a 24 kbp wild-type XbaI fragment and an additional fragment which should be 0.7 kbp longer than the 16.3 kbp fragment characteristic for mouse strain Balb/c, due to the insertion of the neo^r cassette. This latter 17.0 kbp fragment should also hybridize with the neo probe. Furthermore, upon double digestion with XbaI and KpnI we anticipated to see fragments of 17.0 and 11.0 kbp for wt and targeted alleles, respectively. As shown in Fig. **1B**, all expected XbaI and

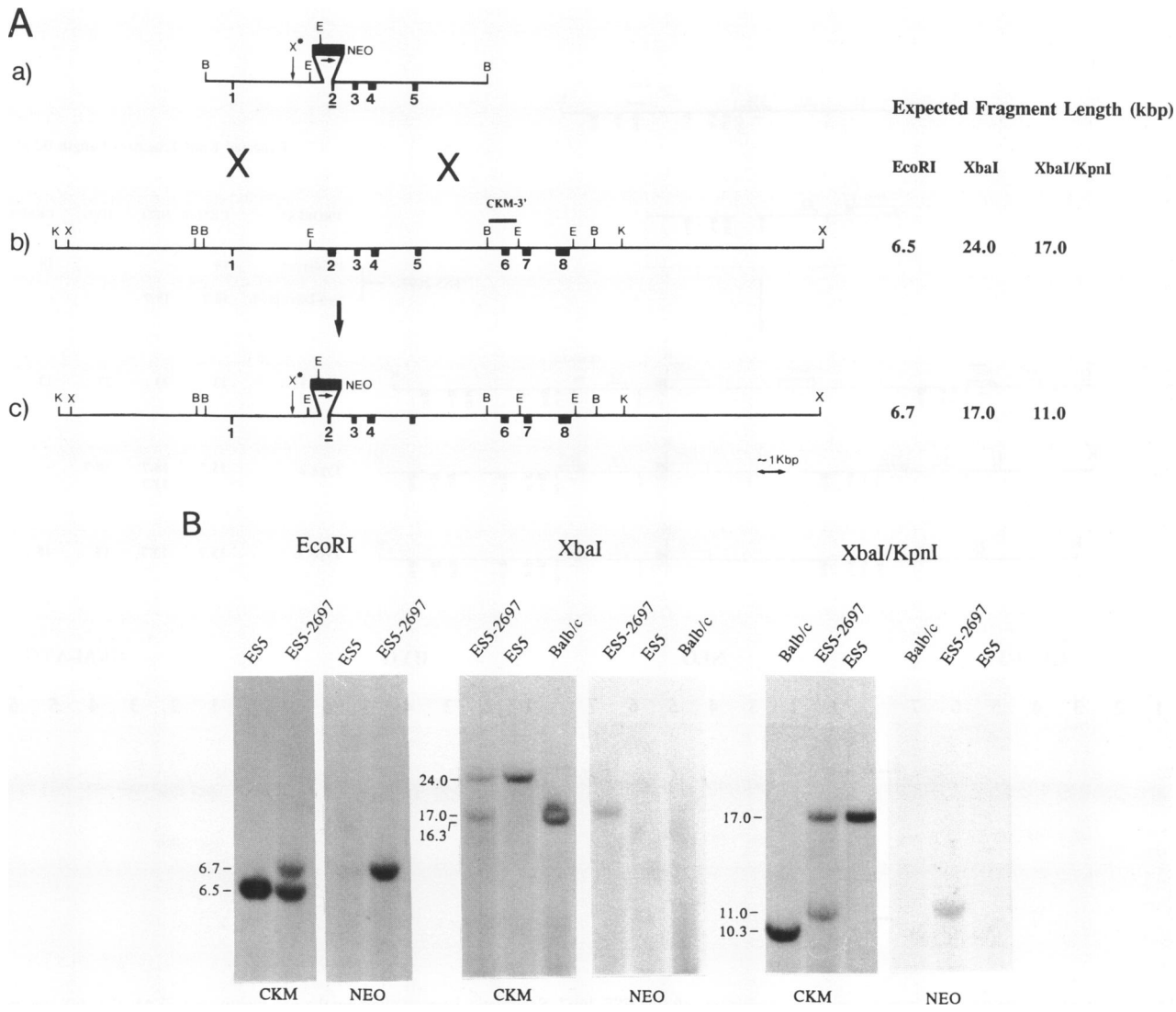

Figure 1A: Schematic diagram showing the targeting vector pRV8.5 (a; plasmid not shown) , the structure of the CKM locus in parental ES5 cells (b) and the predicted structure of the mutated CKM locus (c). Numbers 1−8 denote the exons; the bar indicates the position of the 900 bp BamHI-EcoRI probe fragment of CKM located just outside the targeting vector. As neo probe we used a 900 bp EcoRI-SalI fragment derived from pMC1neo (22). A list of expected fragment sizes hybridizing to the 900 bp CKM probe is given to the right. Abbreviations B, BamHI site; E, EcoRI site; K, KpnI site; X, XbaI site. X* is a polymorphic XbaI site present in intron 1 of the targeting vector (Balb/C mouse-derived CKM gene) and absent in the ES5 cell DNA (129J derived). **B**: Southern-blot analysis demonstrating targeted inactivation of one CKM allele in clone ES5-2697. DNAs were digested with the restriction enzymes indicated and hybridized with ^{32}P-labeled CKM-3′ or neo probe. Fragment sizes are indicated to the left of each panel. Lanes ES5, parental control cell line DNA; lanes ES5-2697, targeted cell line DNA; lanes Balb/C, Balb/C specific spleen DNA.

XbaI/KpnI fragments were detected, giving additional evidence for correct insertional inactivation of one CKM allele in ES5-2697 cells. As an additional check, both the parental ES5 cells and ES5-2697 cells were examined cytogenetically and found to yield fully normal karyotypes (not shown).

Table I.

Vector	Cell line	Treated ES cells, no.	Resistant colonies, no.	Resistant colonies analysed, no.	Targeted colonies, no.
pRV8.5	ES5	29×10^6	440 neo^r	158 neo^r	1
pIV9.0	ES5	24×10^6	900 hygroB^r	191 hygroB^r	0
pIV9.0	ES5-2697	30×10^6	350 hygroB^r	190 hygroB^r	6
pRV8.5	CCE	50×10^6	91 neo^r	74 neo^r	0
		80×10^6	225 neo^r	186 neo^r	0
pIV9.0	CCE	50×10^6	815 hygroB^r	134 hygroB^r	0

In an experiment to see whether the type of ES cell had any influence on targeting efficiency we attempted to repeat the knock-out in CCE cells (see Table 1). No homologous recombinants in 260 DNAs from G418 resistent colonies were observed, thus confirming that primary targeting at the CKM locus in wt ES cells is not a very efficient process.

Enhancer replacement by repeated gene targeting

To interfere with the expression of the CKM gene in a more subtle fashion we constructed an insertion-type of vector, pIV9.0 (12,22), designed to functionally replace the muscle specific 5′ upstream CKM enhancer (E1, position −1100) by the constitutive acting enhancer (EPyF441) from polyoma mutant PyF441 (24) (Figure 2). The targeting DNA contained 9.0 kbp of overall homology to the CKM gene and a hygromycin B resistance (hygroB^r) gene located just upstream of the EPyF441 element

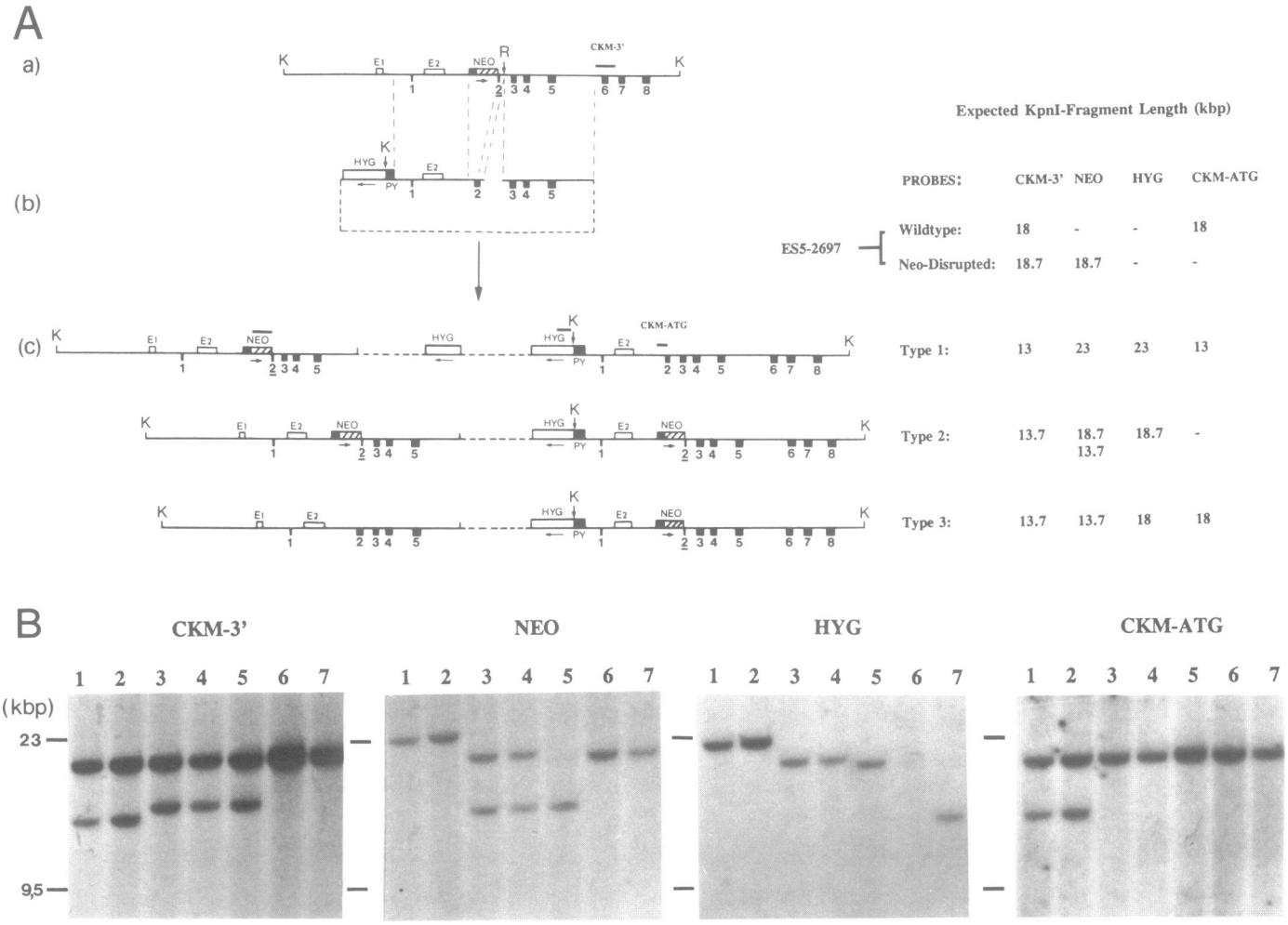

Figure 2A: Second round gene targeting in homologous recombinant cell line ES5-2697. Schematic diagram showing the neo-inactivated CKM allele (a), the insertion vector pIV9.0 (b) and the various structures of the double targeted alleles observed (c). E1 and E2 denote the two enhancer regions in the CKM gene (18, 19). The bars show the location of the CKM, neo and hygroB specific probes. For sake of clarity, the plasmid (broken line) in the targeting vector, is not drawn according to scale. A list of fragment sizes for each of the four probes shown in panel **B** is given to the right. Hybridizing fragment sizes are listed for the wt and the neo-disrupted allele in ES5-2697 and the type 1, type 2 and type 3 doubly targeted alleles. Abbreviations: R, EcoRV; PY, polyoma PyF441 enhancer; HYG, hygromycinB gene; 2, partially deleted exon 2. **B:** Southern analysis of KpnI digests of genomic DNA from five doubly targeted ES5-2697 clones, the original ES5-2697 cell line and a nonhomologous transformant. One additional recombinant displayed the same fragments as those shown in lanes 3 and 4 (type 2). Genomic DNAs were hybridized to the CKM-3′, the neomycin, the hygromycin and the CKM-ATG specific probes as indicated above each panel. DNAs, lane 1, ES5-2697-HB22; lane 2, ES5-2697-HB117 (both type 1); lane 3, ES5-2697-HB88; lane 4, ES5-2697-HB123 (both type 2); lane 5, ES5-2697-98 (type 3); lane 6, ES5-2697; lane 7, ES5-2697-97 (nonhomologous transformant as a control). Fragment sizes are given in kbp.

to allow for double drug selection. We anticipated that targeting might in principle cause two effects. First, the EPyF441 enhancer is known to be active in ES cells (22) and to confer a constitutive—tissue independent—mode of expression to a variety of genes. It is therefore likely, that replacement of the −1100 enhancer region by EPyF441 will yield CKM expression at stages in development where normally CKM is absent. Concomitantly, as we expect the natural CKM E1 enhancer to shift to a position at least 14 kbp further upstream by the insertion event, its influence on the tissue specificity of CKM production, may be lost.

To generate mutant ES cells with either a sole enhancer replacement or with two mutated CKM alleles (i.e. one disrupted allele and one allele with altered expression), EcoRV linearized pIV9.0 DNA was introduced both into wild-type ES cells and into the ES5-2697 cells with the neo-disrupted CKM gene. HygroB^r colonies were selected and targeting events were identified by Southern-blot analysis of KpnI cleaved ES-DNAs with the CKM-3′ probe (Figs. **1** and **2**) used earlier. For the wild-type ES cells, amongst 191 hygroB^r colonies screened, no homologous recombinants were identified. Targeted clones were expected to contain a novel 13.0 kbp sized fragment in addition to the wild-type fragment of 18.0 kbp. In contrast, for the ES5-2697 cells with the neo-inactivated CKM allele, in 6 out of 190 hygroB^r colonies analysed (Table I), a homologous recombination event was observed. Surprisingly, all six clones examined had insertions into the neo-disrupted allele, as can be inferred from the appearance of new KpnI fragments hybridizing with the neo probe (Fig. **2B**). Based on hybridization with the CKM-ATG and hygromycin probes, as well as on additional Southern-blot analyses (not shown) the six independent recombinants can be divided into the three distinct types of clones depicted in Figure **2A**. Two clones, ES5-2697-HB22 and -HB117 designated type 1 mutants, have the body of the CKM gene repaired to normal. In both clones, the novel 13 kbp CKM-3′ positive KpnI fragment spanning the doubly targeted allele yielded also a clear signal with the CKM-ATG probe. As the 18 kbp wt KpnI signal remains unchanged this must mean that the neo insertion must have been corrected and replaced by the original CKM sequence via a targeted correction event presumably involving gene conversion. In addition, both cell lines displayed the planned substitution of the myocyte specific E1 enhancer by the EPyF441 enhancer, an alteration probably due to crossover occurring 3′ of the neo^r gene. Unexpectedly however, we observed in both clones a duplication of plasmid DNA and part of the hygroB^r gene, apparently resulting from an additional gene conversion-like event at the 3′ end of the CKM gene. A small but significant difference in the lengths of the duplicated areas encompassing the hygroB^r genes was observed between both clones (data not shown). The three type 2 clones had the enhancer E1 replaced by EPyF441, and in addition showed a duplication of the neo^r gene. We can only explain these features by assuming that a gene conversion event had introduced the neo^r cassette into the targeting vector before the actual DNA integration, via a crossover 3′ to the neo^r gene, took place. One clone with a type 3 structure as shown in Figure **2A**, was identified. This clone probably resulted from a crossover at the region of homology 5′ upstream of the neo^r gene.

Again, to see whether there was any difference in targeting efficiency between ES cell lines, pIV9.0 DNA was also introduced into ES CCE cells. As expected from the ES5 transfection experiments, no correct targeting events were identified in 134 clones analysed (Table **1**). Possible explanations for this observation, and for the fact that in the second round of targeting only clones with an integration at the neo-disrupted allele were obtained, are discussed below.

Analysis of CK isoenzyme patterns in targeted ES clones

To study the effects of the enhancer substitution on the expression of the CKM gene and to compare this to the situation in the ES5-2697 and wild-type ES cells, the CK isoenzymes were analysed using zymogram-electrophoresis followed by CK activity staining (31, 32). As visualized in figure **3**, both ES5-2697-HB22 and ES5-2697-HB117 (lanes 4 and 6) contain a novel CK-BM heterodimeric isoenzyme activity. This activity is absent from the type 2 clone shown in lane 3 and from the nonhomologous transformant shown in lane 5. Interestingly, formation of BM heterodimers in the type 1 ES cells is favored above production of MM homodimers. Apparently, there is a relatively large pool of free CK-B monomers which titrates the newly formed CK-M monomers and hinders formation of CK-MM. Moreover our results confirm that in ES cells the B- and M-polypeptides can assemble freely in the cell cytoplasm. Hence, there is no need to postulate molecular chaperones (34) or other factors that may influence the specificity of dimerisation.

The CK-BB activity in all four clones analysed was very similar, CK-MM activity in normal or wild-type ES cells was below the level of detection. We conclude that the mere substitution of the CKM E1 upstream enhancer by the PyF441 enhancer, leaving the second E2 enhancer intact, results in the transcriptional activation of the CKM gene in ES cells. The binding of transcriptional activators already present in the ES cells is presumably enhanced by the new arrangement in transcription-factor recognition elements upstream of the CKM cap site. The possibility that further enhancement of expression of the type 1 CKM gene, due to the presence of the E1 enhancer now shifted to approximately 19 kbp upstream of its normal chromosomal location, will result from in vitro or in vivo differentiation of ES5-2697-HB22 or -HB117 into myogenic direction remains a subject for later study.

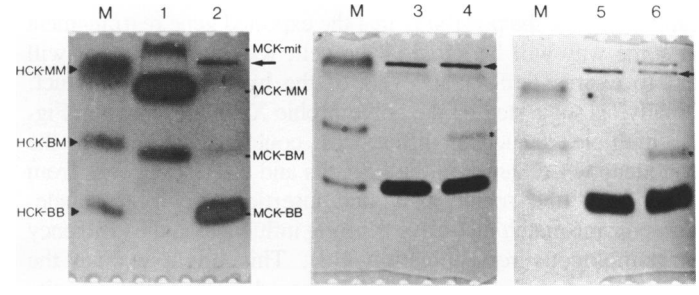

Figure 3: Zymograms of CK isoenzymes in targeted embryonic stem cells and neuro-muscular tissue extracts. M, Human CK markers (Isotrol, Sigma diagnostics); 1, CK isoenzymes in mouse heart extract; 2, CK isoenzymes in mouse brain extract; 3, ES5- 2697-HB88 (type 2); 4, ES5-2697-HB22 (type 1); 5, ES5-2697-HB97 (nonhomologous transformant); 6, ES5-2697-HB117 (type 1). Arrows mark the positions were samples were applied prior to electrophoresis, arrowheads to the left mark the positions of the human CK isoenzymes indicated. Bars indicate the positions of the mouse CK isoenzymes. Asterisks mark the newly synthesized CK-BM isoforms. Significant differences in mobility were observed between human and mouse CK-MM isoenzymes and, less, between CK-BM isoenzymes of both species. Mouse and human CK-BB isoenzymes had identical mobility in this system.

DISCUSSION

Several studies have now documented the potential of targeted gene inactivation in studying the role of specific genes in adult mice or mouse embryonic development (14, 15, 16). Usually gene inactivation is brought about by inserting a neo^r cassette in the coding portion of a gene. However, in cases where one can predict lethality in homozygous null mutants, targeted introduction of more specific mutations may be desirable. In this paper we describe experiments designed to explore the potential of homologous recombination for our studies into the role of subcellular and tissue-specific isoenzyme distribution of creatine kinases, using the CKM gene as a model. We have shown that it is possible to use homologous recombination techniques not only to render one CKM allele non-functional but also to create a gain of function mutation by promoter modulation. To obtain these latter changes, we used a strategy that involved the targeted replacement of the tissue-specific CKM enhancer E1 by the mutant polyoma enhancer EPyF441. Our experimental design involved use of dual drug selection as recently also reported by Te Riele et al. (35). Remarkable is our observation, that not only the second round of homologous recombination on our primary targeted ES cell line ES5-2697 was rather efficient but that all secondary targeted clones (6 out of 190 tested) resulted from recombination events at the neo-disrupted CKM allele. There may be several explanations for the sole identification of clones that are targeted twice at the same allele. Firstly, the neo-disrupted allele could be a better substrate for targeted insertion than the wild-type CKM allele for the reason that the disruption may have induced a more 'open' chromatine structure resulting in a greater accessibility for enzymes of the recombination machinery (14). Perhaps the mere presence of bacterial DNA or more likely, the EPyF441 enhancer in the neo^r cassette could be responsible for these putative changes in chromatin topology (36). Alternatively, the increased transcriptional activity at the neo^r gene may have had some effect (see also ref. 37). Secondly, the unique integration at the neo-disrupted CKM locus may not have been caused by increased and preferential recombination at the neo-disrupted allele but by the inability of the insertional vector to target the wild-type locus. Because, with the hygromycin vector, we did not observe targeted insertion in wild-type ES cells, we cannot exclude the possibility that the expected gene rearrangment in some way will be lethal to the cell, will be unstable or will fail to express sufficient levels of the hygroB^r gene product. Finally, as suggested by the polymorphic XbaI site shown in Fig. 1, multiple sequence differences could exist beween the endogenous CK genes in the ES cells and the CKM genes from which both the replacement and insertion vector are made. Basepair mismatch may have a strong influence on the efficiency of homologous recombination (48). This might explain the stronger preference for targeting the neo-disrupted allele in spite of the fact that the overall length of homology between pIV9.0 and the mutated allele is slightly reduced as compared to the situation at the wt CKM allele. Allele specific targeting in the second round of transformation as observed for the CKM gene has not been observed in a recent study involving the consecutive inactivation of the pim-1 gene (35). Perhaps, as also primary targeting frequencies vary widely between different regions of the genome, we may assume that the difference in allelic preference for second round targeting as observed for pim-1 and CKM genes may equally well be locus or gene dependent. Though indeed, recent evidence has shown that identical alleles at certain loci do sometimes behave functionally different depending on the parental sex (38) the discrepancy between both findings is equally likely to result from differences in vector design (i.e. replacement versus insertion type vectors) or experimental technique.

Whatever the reason, from our observations we may conclude that primary CKM-targeted clones are relatively hard to obtain compared to secondary CKM-targeted clones. Therefore, deliberate use of repeated targeting in cell line ES5-2697, may provide an easier route for creating other subtle mutations that affect CKM expression. CKM mRNA transcription and splicing processes are perhaps the most amenable for manipulation using the schemes as presented here. While the observed difference in allelic targeting needs to be shown for other genes, our findings may have consequences for experimental design in similar studies. Firstly, it may sometimes be difficult to achieve targeting of both alleles in cases where it is not possible or desirable to have germ line transmission. Secondly, for certain genes—as for the CKM gene shown here—one may find more expedient routes for creating allelic mutations by deliberately using repeated targeting. Thus, the first targeting serves to make the gene more accessible to subsequent modifications.

Surprisingly, none of the second round targeted CKM structures were of the form predicted by a single crossover near the EcoRV site, but all resulted from more complex recombination events, presumably involving gene conversion in at least 4 out of 6 cases. Though small sized deletions have been found to be corrected in gene conversion events by others (39−41), duplications of approximately 1 to 4.5 kbp long stretches of dishomologous DNA have not been observed before except for in one case (42). One striking observation from our results is that out of the six clones obtained, in two, ES-2697-HB22 and -HB117, the secondary targeting event that led to repair of the body of the gene, has completely restored the potential to encode biological active CKM-protein subunits. Previously, gene targeting associated with gene repair has been demonstrated for other genes (12, 41, 43). As shown in Figure 3, biologically active CK-BM heterodimers with a mobility similar to the normal mouse BM isoenzyme, were formed. It should now be possible to begin studies into the biological consequences of CKM null and up- mutations for embryonic development and intracellular distribution. From analogy with transgenic experiments giving ectopic CKB expression (44, 45) we anticipate that 'abnormal' CKM expression should be compatible with life. Furthermore, previously our group had identified a 19/X chromosome translocation in a human female patient that had rendered one of the two CKM alleles non-functional (46). From this, and from the fact that CKM expression occurs late in embryogenesis in skeletal and cardiac myocytes, where it functionally replaces or is coexpressed with CKB, we expect inactivation of one CKM allele not to be embryonic lethal. However, because genomic imprinting is known to exist in the chromosomal region in which the mouse CKM gene is located (38, 47) , we are aware of the fact that biological effects of single CKM allele mutations may still be hard to predict. More definite answers can therefore only be expected from a detailed study of the behaviour of ES5-2697 and ES5-2697-HB22 cells in mouse embryonic development now in progress. From recent experiments, involving reinjection of both the primary targeted ES5-2697 cells as well as the secondary targeted ES5-2697-HB22 cells into blastocysts of C57B6 mice, we may already conclude that both cell types have retained their ability to contribute to tissue formation in chimeric mice. The mice obtained are now being bred to asses germ line transmission of the altered genes.

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