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Targeting of the creatine kinase M gene in embryonic stem cells using isogenic and nonisogenic vectors

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ABSTRACT

Replacement vectors with genomic DNA originating from different mouse strains were used to introduce site-specific mutations into the creatine kinase M (CKM) gene of mouse embryonic stem (ES) cells. Here we demonstrate that in mouse strain 129-derived ES cells, the gene is at least 25-fold more efficiently targeted with an isogenic, 129-derived vector (129-pRV8.3) than with a nonisogenic, BALB/c-specific vector (BALB/c-pRV8.3). The two targeting constructs were identical except for allelic differences which were typed by partial sequencing. These included base pair mismatches (2%) and a polymorphic [GTC]-repeat length variation. Both in separate transfections as well as in cotransfections with mixed vectors, homologous disruption of the CKM gene resulted uniquely from the 129-isogenic DNA. Our data confirm earlier observations on requirements for homologous recombination in pro- and eukaryotic systems and indicate that targeting of the CKM locus is highly sensitive to small sequence differences between cognate segments in the endogenous and incoming DNA.

INTRODUCTION

Gene targeting, homologous recombination between newly introduced DNA and chromosomal DNA, has been used to alter specific chromosomal loci in murine ES cells. These ES cells may subsequently be used to introduce the mutation into the germ line of mice to study the phenotypic consequences at the animal level (1–7). However, since random recombination is about 30- to 40000-fold more frequent than homologous recombination in mammals (8), the identification of targeted cells can become a laborious task. Better understanding of the various parameters that influence the frequency of gene targeting may simplify and economize the experiments that require ES cells with site-specific mutations.

Several studies have illustrated that the targeting frequency in mammalian systems is dependent on homology length (9–11). Furthermore, the topology of the incoming DNA may play a role as very different targeting frequencies between an insertion and a replacement vector containing identical homologous sequences have recently been observed (8). Besides homology length and vector configuration, base pair variation may effect the rate of homologous recombination. It has been shown that the homologous recombination machinery of prokaryotes (*Escherichia coli*) is very sensitive to base pair mismatches between the substrates (12). In *E.coli*, the crucial factor that determines the recombination efficiency appears to be the length of the uninterrupted stretches of homology (12, 13). Likewise, in mammalian cells, it has been shown that the efficiency of intrachromosomal recombination is determined by the amount of uninterrupted homology available and does not depend on the total number of mismatches within a given interval of DNA (14). We have been focussing on the use of targeted mutagenesis of members of the creatine kinase (CK) gene family (4 genes encoding 4 CK isoenzyme subunits) to study the processes involved in the generation and use of cellular ATP (for review see 15). In these studies we encountered conspicuous but unexplained differences in targeting frequencies for different CK alleles (16). In an ES cell targeting experiment one CKM allele was completely refractory to homologous recombination whilst the other, previously modified, allele was targeted at a high frequency. Furthermore, in a preliminary experiment involving the creatine kinase B (CKB) gene, site-specific disruption in mouse strain 129-derived ES cells was much more efficient with a vector originating from the same mouse strain than with a vector containing DNA from mouse strain CBA (17). However, direct comparison of the targeting frequencies was difficult since there were not only strain specific DNA sequence heterologies between the two vectors but also small differences in homology length and position of the neomycin resistance cassette. These observations have recently gained a more solid basis through the work of Te Riele et al. who demonstrated the importance of the use of isogenic DNA constructs for targeting the retinoblastoma susceptibility (RB) gene in 129-derived E14 ES cells (18).

As a further step towards understanding of homology requirements of targeted mutagenesis we report here on a systematic study of the effects of homology between the incoming DNA and the target cell genomic DNA using strain specific

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vectors and the CKM gene of mouse as a model. In separate as well as mixed assays, the isogenic 129-derived vector DNA showed by far the highest targeting efficiency.

MATERIALS AND METHODS

Construction of vectors

The replacement vector 129-pRV8.3 was derived from a 9.0 kilobase pairs (kbp) genomic BamHI fragment spanning exons 1–5 of the CKM gene, that originated from a mouse strain 129Sv/E genomic DNA library in phage EMBL3 (17). A 0.9 kbp EcoRI-EcoRV fragment, encompassing exon 2 and part of introns 1 and 2, was removed and replaced by a 2.0 kbp hygroB^r cassette (16). Furthermore, a 2.0 kbp HSV-tk cassette was attached to the 3′ end of the CKM gene. In this cassette the polyoma enhancer PyF441 is driving the expression of the tk gene which carries the polyadenylation signal of pMCneopolyA (9). BALB/c-pRV8.3 was constructed as 129-pRV8.3 except that the 9.0 kbp BamHI fragment originated from a mouse strain BALB/c genomic DNA library (16). To enable the typing of targeting events by Southern blot analyses, the unique XhoI site in the 5′ polylinker sequence of the hygroB^r cassette in BALB/c-pRV8.3 was replaced by an EcoRV site. To this end, DNA was digested with XhoI, filled in with dNTPs using Klenow DNA polymerase and ligated to EcoRV linkers (see Fig. 2). The targeting plasmids were sequenced using the supercoil DNA sequencing method (19).

Electroporation and tissue culture

The DNA was prepared for transfection by alkali lysis (20), linearized with restriction enzyme BamHI, phenol-chloroform extracted, ethanol precipitated and suspended in H_2O at a concentration of 0.5–1.0 μg/μl. Targeting vector DNA was introduced into AB-1 cells (4) (kindly provided by Dr. A.Bradley, Baylor College of Medicine, Houston, USA) by electroporation with a TA750 transfection apparatus (Krüss GmbH Hamburg, Germany). Batches of about 4–8.10^6 AB1 cells were suspended in 0.4 ml electroporation buffer [10 mM potassium phosphate (pH 7.1)/0.28 M Sucrose/1 mM $MgCl_2$/200 μg/ml BSA (Boehringer Mannheim GmbH)] and mixed with 10 μg/ml vector DNA. For cotransfection of 129-pRV8.3 and BALB/c-pRV8.3, cells were mixed with 5 μg/ml of each plasmid (10 μg/ml total). The cells were given 24 pulses [17 μs each, 1.5 kV/cm], incubated for 1 min at room temperature and then plated onto a 9 cm tissue culture dish carrying SNLH9 feeder cells in 10 ml of medium (Dulbecco's modified Eagle's medium with 15% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM β-mercaptoethanol). SNLH9 feeder cells were clonally derived from cell line SNL76/7 (4) after transfection with vector pG3-hygroB DNA (16) containing the hygroB^r gene (Deursen van, unpublished). 24 hr after electroporation ES cells were refed with medium containing 140 μg/ml hygromycin B (dry powder, Sigma) and 0.2 μM FIAU (1-[2-deoxy, 2-fluoro-β-D-arabinofuranosyl, a kind gift of Bristol Myers). Ten days after transfection individual colonies were picked and expanded on 60% (v/v) BRL-conditioned medium (16) (to remove SNLH9 feeder cells) for DNA preparation and storage. To determine the negative selection enrichment factor of 129-pRV8.3 and BALB/c-pRV8.3, cells were first grown for ten days on medium containing 140 μg/ml hygromycin B only and then colonies were scored. Subsequently, individual colonies were grown on hygromycin B plus FIAU medium for 1 week and then scored again.

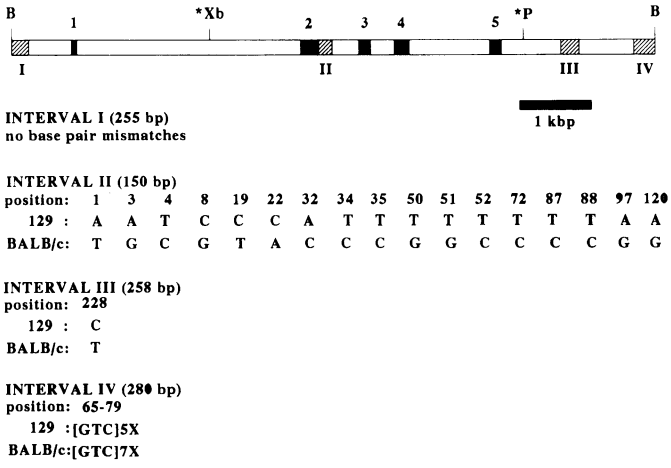

Figure 1. Partial DNA sequence comparison of the CKM genes from mouse strains 129 and BALB/c. Sequence mismatches identified within four randomly chosen DNA intervals (I–IV, hatched boxes) are given underneath the 9.0 kbp BamHI fragment used for construction of the targeting vectors 129-pRV8.3 and BALB/c-pRV8.3 (see Fig. 2). The black boxes mark the positions of the CKM exons 1–5. Polymorphic restriction sites XbaI and PstI, which are present only in the BALB/c derived CKM allele are denoted by *Xb and *P. In all four DNA regions sequenced nucleotide position 1 is located at the 5′ end of each interval.

Southern blot analyses of targeted clones

Genomic DNAs were subjected to restriction enzyme digestion with KpnI, EcoRV and XbaI/XhoI. Approximately 2.0 μg of each digest was fractionated on a 0.7% agarose gel, transferred to Hybond (Amersham), and hybridized with ^{32}P-labeled probes as previously described (16). Probe A, a 900 bp BamHI-EcoRI fragment (Figure 2a), has no overlap with sequences present in 129- and BALB/c-pRV8.3. Probe B, a 1195 bp PstI-BglII fragment derived from pECV6 (21), was used as a hygroB specific probe (see Fig. 2). After hybridization, blots were washed at a final stringency of 0.05× SSC, 0.1% SDS at 65°C.

RESULTS

Dishomology between the CKM genes of mouse strains 129 and BALB/c

In order to assess the influence of sequence divergence between targeting vector and endogenous DNA in a systematic manner we constructed a set of highly similar isogenic and nonisogenic knock-out vectors for the murine CKM gene. Initially, from Southern blot analyses of CKM gene regions of mouse strains 129 and BALB/c genomic DNAs, XbaI (16) and PstI restriction fragment polymorphisms (see Fig. 1) were observed. To study the extent and the nature of the dishomology more precisely, we partially sequenced randomly distributed intervals of the 129- and BALB/c-derived 9.0 kbp CKM fragment used for construction of the strain specific targeting vectors. The comparative sequence analysis is illustrated in Figure 1. Of the four DNA regions sequenced only interval I (255 bp) shows perfect homology between 129 and BALB/c. In interval II (150 bp) 17 single nucleotide mismatches (11%) were found. In interval III (258 bp) and interval IV (280 bp) 1 nucleotide substitution and a polymorphic [GTC]-repeat were observed respectively. From

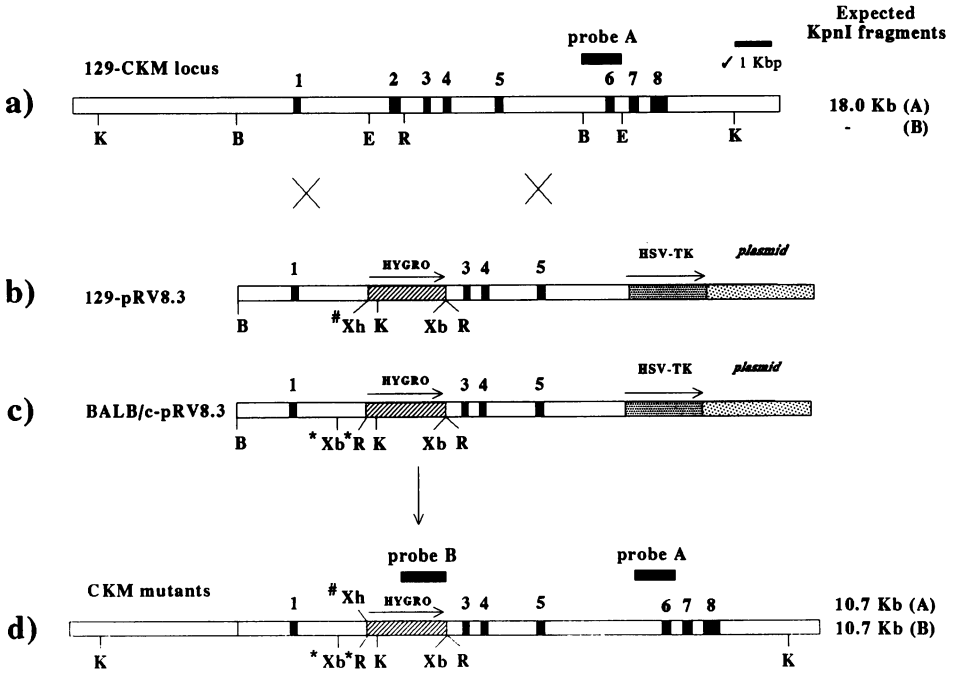

Figure 2. Schematic diagram showing (a) the structure of the CKM locus in parental AB-1 cells (b) the mouse strain 129-derived targeting vector 129-pRV8.3 (c) the BALB/c-derived vector BALB/C-pRV8.3 and (d) the predicted structure of the CKM locus after targeted mutagenesis with 129-pRV8.3 or BALB/c-pRV8.3. Numbers 1–8 denote the exon sequences, the bars indicate the positions of the probes A and B. A list of KpnI fragment sizes hybridizing to both probes A and B is given to the right. Abbrevations B, BamHI; E, EcoRI; K, KpnI; R, EcoRV; Xb, XbaI. The EcoRV and XbaI sites only present in BALB/c-pRV8.3 are marked by *, and the XhoI site diagnostic for 129-pRV8.3 is denoted by #Xh.

Table 1. A. Targeting of the CKM gene in AB-1 cells using isogenic (129-pRV8.3) and nonisogenic (BALB/c-pRV8.3) plasmid vectors.

Exp. no.	Targeting DNA	Electroporated cells, no.	HygroB^r/FIAU^r clones, no.	HygroB^r/FIAU^r clones sreened, no.	Targeted clones, no.
1	129	20×10^6	158	110	18
2	129	30×10^6	150	99	7
3	BALB/c	60×10^6	204	141	0
4	BALB/c	20×10^6	148	73	0
5	BALB/c	30×10^6	156	143	0
6	BALB/c-129 (1:1)	30×10^6	200	139	12
7	BALB/c-129 (1:1)	30×10^6	144	105	3

B. Frequencies of conversion of AB-1 cells to hygroB resistance and FIAU-enrichment factors

Targeting DNA	Electroporated cells, no.	HygroB^r clones, no.	Frequency of hygroB^r clones	HygroB^r/FIAU^r clones, no.	Enrichment factor
129	8×10^6	301	3.8×10^{-5}	126	2.4
BALB/c	8×10^6	286	3.6×10^{-5}	114	2.5

these data we estimate that on average approximately 2% allelic nucleotides exist between the 129-CKM and the BALB/c-CKM loci.

Strain specific targeting vectors

To measure the frequencies of homologous recombination with isogenic and nonisogenic vectors, we used the gene targeting strategy as previously outlined by Mansour et al. (22). The two replacement-type targeting vectors, 129-pRV8.3 and BALB/c-pRV8.3 (see Fig. 2) contain a 9.0 kbp segment of the CKM gene flanked by BamHI sites which are conserved between the mouse inbred strains 129 and BALB/c. In these 9.0 kb fragments a 0.9 kbp EcoRI-EcoRV fragment was replaced by a hygroB^r cassette (16). In addition, to enable selection against random integration events, a HSV-tk gene was introduced at the 3′ end of the CKM homology (22). This tk gene is driven by the same promoter and polyoma enhancer as the hygroB^r gene. The colinearity of both targeting constructs was confirmed by restriction enzyme analysis and DNA sequence analysis.

Testing of vector efficiency in separate transfection assays

Firstly the two BamHI linearized targeting vectors were *separately* introduced into mouse strain 129-derived AB-1 cells. Simultaneous selection in hygromycin B and the pyrimidine

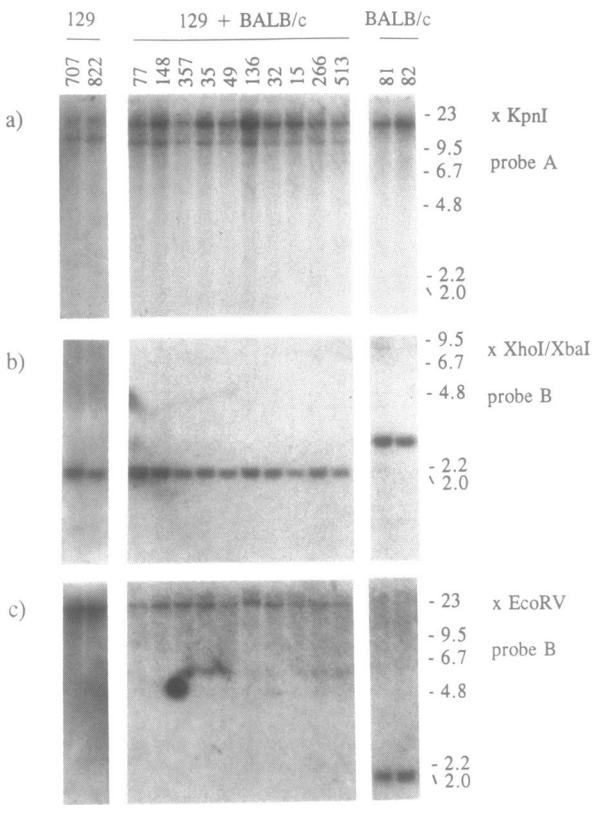

Figure 3. Southern blot analyses of DNA from targeted and nontargeted ES cell lines. (a) KpnI digested DNAs hybridized to probe A (CKM). (b) XbaI and XhoI digested DNAs probed with probe B (hygromycin) and (c) EcoRV digested DNAs also hybridized to probe B. At the right site of each panel the positions of DNA marker fragments (HindIII digested phage lambda DNA) are indicated in kbp. Numbers on top of the upper panel refer to the various AB-1 ES clones selected: 707 and 822, two 129-pRV8.3 targeted clones from the *separate* transfection experiments; 77, 148, 357, 35, 49, 136, 32, 15, 266 and 513, homologous recombinants derived from *cotransfections* of vectors 129-pRV8.3 and BALB/c-pRV8.3, and 81 and 82, clones with random integrations of BALB/c-pRV8.3 from a *separate* transfection experiment.

derivative FIAU yielded colonies which were clonally expanded, and used for isolation of genomic DNA to identify homologous recombinants by Southern blot analyses. The results of the various experiments are summarized in Table 1a. With a CKM specific probe located outside the introduced DNA fragment (probe A, see Fig. 2), targeted clones were expected to yield a wildtype 18.0 kbp fragment as well as a mutant 10.7 kbp fragment. In two experiments with 129-pRV8.3, we indeed identified a total of 25 independent homologous integration events out of 209 colonies screened (frequency 12%). The analysis of two of these recombinants is shown in Fig. 3a, lanes 707 and 822. In contrast, in three individual experiments with construct BALB/c-pRV8.3, amongst 357 hygroB^r and FIAU^r colonies analyzed in total, no homologous recombinants were observed.

In addition, we tested if disparities in the targeting efficiency for 129-pRV8.3 and BALB/c-pRV8.3 could be caused by vector specific differences in the frequency of conversion of cells to hygromycin B resistance or the FIAU-enrichment factor. As seen in Table 1b, both parameters are comparable for 129-pRV8.3 and BALB/c-pRV8.3, and therefore do not contribute to the observed difference in targeting frequency.

Testing of vector efficiency by cotransfection

Previous reports illustrate that the homologous recombination frequency of individual targeting vectors can vary considerable between separate experiments (1, 3, 23, 24). Therefore, to study the targeting capacity of 129-pRV8.3 and BALB/c-pRV8.3 free from any bias imposed by experimental variation, we *cotransfected* equimolar amounts of both linearized vectors into the AB-1 ES cells. The subsequent selection of doubly resistant colonies and screening of homologous recombinants were carried out as mentioned above. Two separate experiments yielded a total of 15 homologous recombinants out of 244 colonies screened, giving a targeting frequency of about 1 in 16 hygroB^r/FIAU^r clones (see Table 1a and Fig. 3a). To identify whether the 129-pRV8.3 or BALB/c-pRV8.3 vector DNA had been involved in the recombination event, Southern blot analyses were performed for every individual clone using the XbaI/XhoI and EcoRV diagnostic restriction sites present in the polylinker DNA flanking the hygroB^r gene of each vector (see Fig. 2). Thus, DNA samples of all individual targeted clones were digested with simultaneously XbaI and XhoI or EcoRV and blotted and hybridized with probe B, a hygroB^r gene specific probe. In those clones in which the substrate involved in the targeted disruption of the CKM gene is 129-pRV8.3, probe B will detect a 2.0 kbp XhoI/XbaI fragment. In the clones targeted by BALB/c-pRV8.3 a 2.0 kbp EcoRV fragment will become visible. All 15 homologous integrants displayed the 2.0 kbp XhoI/XbaI band whereas the 2.0 kbp EcoRV fragment was absent and replaced by a band of more than 23 kbp, indicating that in every recombination event vector 129-pRV8.3 was involved. The Southern blot analyses of 10 of these 15 clones are given in Figure 3.

To verify further the discriminative abilities of our procedure, also two nonhomologous BALB/c-pRV8.3 integrants of experiment 3 (see Table 1a) were analyzed. As expected, in both clones the diagnostic 2.0 kbp EcoRV fragment was present whilst the 2.0 kbp XhoI/XbaI band was absent (Fig. 3 lanes 81 and 82), demonstrating the reliabili ty of the diagnostic approach.

Finally, as an additional check for the integrative capacity of the vector DNAs proper, we selected a number of nontargeted clones of experiments 6 and 7 (see Table 1a) carrying a single vector DNA integration and identified the mouse strain origin of the construct involved. Because equimolar mixtures of isogenic and nonisogenic DNA were used to transfect the ES cells, 129-pRV8.3 and BALB/c-pRV8.3 DNA integrations were expected to be equally distributed amongst clones. Southern blot analyses of 18 clones revealed that 10 clones had a single integration of 129-pRV8.3 and 8 originated from BALB/c-pRV8.3 (data not shown), indicating that experimental conditions indeed had the anticipated results.

DISCUSSION

The development of more efficient selection (22, 25) and screening (26, 27) procedures and of improved targeting vectors (10, 11) has considerably simplified the identification of targeted clones in ES cell mutagenesis experiments. Still the frequencies of gene targeting are rather low in the various experiments that have been reported until now. Further progress in the technology may therefore come from a better understanding of the aspects that influence the efficiency of homologous recombination. With the results presented here we underscore the need to explore the

homologous recombination potential of isogenic targeting constructs in future experiments. Though our tests are based on the use of only one gene, i.e. the CKM gene located on mouse chromosome 7 (28), they demonstrate that targeted gene disruption in ES cells derived from mouse strain 129 can be obtained at high frequency (ratio homologous to random integration, 1 to 8) provided that a syngenic targeting construct, derived from strain 129, is used. With the nonisogenic BALB/c-derived vector no targeting events were found in 357 clones analyzed. The most compelling evidence for the sensitivity of targeted recombination to base pair mismatches, however, comes from the experiment where both types of constructs were transfected simultaneously. In this experiment we attempted to maximally suppress the eventual influence of cell culture condition, electroporation bias or selection regime. Even here, the isogenic 129-derived vector was the only construct involved in homologous integration. Because the vectors were designed such that they differed only at the region of homology, these findings indicate that the machinery involved in targeted recombination is sensitive to subtle sequence differences. At present we do not know whether there are any effects caused by the allelic sequence differences being not evenly distributed across the locus. Interval I, just upstream of the first exon was exactly identical in both strains. In interval II, a region situated in the gene's second intron, however, a conspicuous high number of base pair differences was seen (17 out of 150). In intervals III and IV, both located in the 5th intron, only a nucleotide difference and a simple sequence motif polymorphism were found. In spite of these patchwise dissimilarities the deduced overall sequence divergence (2%) for the BALB/c and 129 CKM alleles is only slightly higher than previously reported for the CBA and 129 alleles of CKB (another member of the mouse CK gene family) (17). Also for the RB genes of mouse strains of BALB/c and 129 only about 0.7% divergence was reported (18). Still, many other allelic mouse loci have to be sequenced and tested in ES cell mutagenesis before we will be able to appreciate the influence of the overall sequence context, and individual base transversions or transitions therein, on targeting frequency.

Our CKM data presented here are remarkably similar to the recent findings of te Riele et al. (18). They showed that homologous recombination at the RB gene in 129-derived ES cells was 20-fold more efficient with an isogenic DNA construct than with a BALB/c construct. However, the differences in targeting frequencies for the RB (1 in 3) and the CKM gene (1 in 19 without FIAU-enrichment) indicate that parameters other than homology may influence the gene targeting frequency. Such parameters involved may be the chromosomal location of the target gene, the entire length of the homologous segments (9–11), the topology of the targeting vector (8), the expression level of the selection marker (18) and its position within the homologous DNA segment (2, 29). This may explain why high rates of gene targeting, like those reported here, have been reported by others using nonisogenic DNA vectors (2, 30).

In an earlier study, Waldman and Liskay have shown that in mammalian cells the frequency of *intrachromosomal* recombination events is not determined by the number of mismatches in a given DNA interval but by the amount of uninterrupted homology available. Between 134 to 232 bp of perfect homology seems to be required for efficient homologous recombination. These authors also demonstrated that initiation of intrachromosomal recombination is strongly reduced by DNA mismatches, whereas propagation and termination appear to be less sensitive (14). Therefore, one of the many possible explanations for the efficient targeting with nonisogenic vectors mentioned above could be the presence of large uninterrupted homology at the positions of recombination initiation. It is however hard to predict whether the same extend of uninterrupted DNA homology is necessary and required for efficient *gene disruption* for it is presently unknown whether the same mechanism is involved in the intrinsically different processes of recombination between two chromosomal loci and between chromosomal loci and exogenous DNA vectors. Previously reported data showed that *extrachromosomal* recombination in mammalian cells is relatively insensitive to base pair mismatches, illustrating the individual responses of different types of recombination processes to DNA heterologies (31).

Furthermore, Wahls et al. (32) have shown that the presence of [TG] repetitive elements can stimulate homologous recombination between two plasmid substrates in human tissue culture cells. Again if we can extrapolate these findings to the events involved in gene targeting, simple sequence motif disparities such as the [TG]-repeat length polymorphism observed in CKB (17) and RB alleles (18), and perhaps the [GTC] motif polymorphism in the CKM alleles (Figure 1), may have an even stronger influence on targeting frequencies than simple single nucleotide polymorphisms proper.

Our findings may also have particularly important consequences for vector design for targeting in ES cell lines other than mouse ES cells. For example, targeted integration of selectable markers in somatic cell hybrids, for chromosome tagging as a first step in positional cloning approaches, and for the use in reverse genetics studies in tissue culture cells has been proposed (33). Simple sequence motifs on average are present once in every 1000 to 10000 bp of genomic DNA of these mammalian cells (34). Moreover, in outbred situations like in humans, single nucleotide polymorphisms are present at an estimated frequency of once every 200 bp, a rate that may even exceed the allelic variation in inbred mice as reported here. Therefore, the source of the DNA as starting material for vector construction may become a highly critical issue.

Though we cannot overinterpret our results as large differences in recombination frequencies between isogenic and nonisogenic DNA targeting constructs so far have only been convincingly demonstrated for the RB gene (18) and here for the CKM gene, we feel that the use of DNA vectors with perfect homology may indeed facilitate the identification of targeted clones in reverse genetics approaches, in general.

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REFERENCES

1. Thompson, S., Clarke, R., Pow, A. M., Hooper, M. L. and Melton, D. W. (1989) Cell 56, 313–321.
2. Zijlstra, M., Li, E., Subramani, S. and Jaenisch, R. (1989) Nature 342, 435–438.
3. DeChiara, T. M., Efstratiadis, A. and Robertson, E. J. (1990) Nature 345, 78–80.

4. McMahon, A. P. and Bradley, A. (1990) Cell 62, 1073–1085.
5. Thomas, K. R. and Capecchi, M. R. (1990) Nature 346, 847–850.
6. Koller, B. H., Marrack, P., Kappler, J. W. and Smithies, O. (1990) Science 248, 227–1230.
7. Soriano, P., Montgomery, C., Geske, R. and Bradley, A. (1991) Cell 64, 693–702.
8. Hasty, P., Rivera-Pérez, J., Chang, C. and Bradley, A. (1991) Mol. Cell. Biol. 11, 4509–4517.
9. Thomas, K. R. and Capecchi, M. R. (1987) Cell 51, 503–512.
10. Schulman, M. J., Nissen, L. and Collins, C. (1990) Mol. Cell. Biol. 10, 4466–4472.
11. Hasty, P., Rivera-Pérez, J. and Bradley, A. (1991) Mol. Cell. Biol. 11, 5586–5591.
12. Shen, P. and Huang, H. V. (1986) Genetics 112, 441–457.
13. Watt, V. M., Ingles, C. J., Urdea, M. S. and Rutter, W. J. (1985) Proc. Natl. Acad. Sci. USA 81, 4768–4772.
14. Waldman, A. S. and Liskay, R. M. (1988) Mol. Cell. Biol. 8, 5350–5357.
15. Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. and Eppenberger, H. (1992) Biochem. J. 281, 21–40.
16. Deursen van, J., Lovell-Badge, R., Oerlemans, F., Schepens, J. and Wieringa, B. (1991) Nucl. Acids Res. 19, 2637–2643.
17. Deursen van, J., Schepens, J., Peters, W., Meijer, D., Grosveld, G., Hendriks, W. and Wieringa, B. (1992) Genomics 12, 340–349.
18. Te Riele, H., Robanus Maandag, E. and Berns, A. (in the press) Proc. Natl. Acad. Sci.
19. Hattori, M. and Sakaki, Y. (1986) Anal. Biochem. 152, 232–238.
20. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: A Laboratory Manual (2nd edition). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
21. Belt, P. B., Groeneveld, H., Teubel, W. J., Putte, v. d. P, and Backendorf, C. (1989) Gene 84, 407–417.
22. Mansour, S. L., Thomas, K. R. and Capecchi, M. R. (1988) Nature 336, 348–352.
23. Charron, J., Malynn, B. A., Robertson, E. J., Goff, P. G. and Alt, F. W. (1990) Mol. Cell. Biol. 10, 1799–1804.
24. Stanton, B. R, Reid, S. W. and Parada, L. F. (1990) Mol. Cell. Biol. 10, 6755–6758.
25. Jasin, M. and Berg, P. (1988) Genes and Development 2, 1353–1363.
26. Kim, H-S. and Smithies, O. (1988) Nucl. Acids Res. 16, 8887–8903.
27. Ramírez-Solis, R., Rivera-Pérez, J., Wallace, J. D., Wims, M., Zheng, H. and Bradley, A. (1992) Anal. Biochem. 201, 331–335.
28. Saunders, A. M. and Seldin, M. F. (1990) Genomics 6, 324–332.
29. Koller, B. H. and Smithies, O. (1989) Proc. Natl. Acad. Sci. USA 86, 8932–8935.
30. Riele te, H., Robanus-Maandag, E., Clarke, A., Hooper, M. and Berns, A. (1990) Nature 348, 649–651.
31. Waldman, A. S. and Liskay, M. R. (1987) Proc. Natl. Acad. Sci. USA 84, 5340–5344.
32. Wahls, W. P., Wallace, L. J. and Moore, P. D. (1990) Mol. Cell. Biol. 8, 5350–5357.
33. Dorin, R. D., Inglis, J. D. and Porteous D. J. (1989) Science 243, 1357–1360.
34. Epplen, J. T., Ammer, H., Kammerbauer, C., Schwaiger, W., Schmid, M. and Nanda, I. (1991) Adv. Molec. Genet. 4, 301–310.