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# Recycling Selectable Markers in Mouse Embryonic Stem Cells

ALEJANDRO ABUIN[1] AND ALLAN BRADLEY[1,2]*

*Department of Molecular and Human Genetics[1] and Howard Hughes Medical Institute,[2] Baylor College of Medicine, Houston, Texas 77030*

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**As a result of gene targeting, selectable markers are usually permanently introduced into the mammalian genome. Multiple gene targeting events in the same cell line can therefore exhaust the pool of markers available and limit subsequent manipulations or genetic analysis. In this study, we describe the combined use of homologous and CRE-*loxP*-mediated recombination to generate mouse embryonic stem cell lines carrying up to four targeted mutations and devoid of exogenous selectable markers. A cassette that contains both positive and negative selectable markers flanked by *loxP* sites, rendering it excisable by the CRE protein, was constructed. Homologous recombination and positive selection were used to disrupt the *Rep-3* locus, a gene homologous to members of the *mutS* family of DNA mismatch repair genes. CRE-*loxP*-mediated recombination and negative selection were then used to recover clones in which the cassette had been excised. The remaining allele of *Rep-3* was then subjected to a second round of targeting and excision with the same construct to generate homozygous, marker-free cell lines. Subsequently, both alleles of *mMsh2*, another *mutS* homolog, were disrupted in the same fashion to obtain cell lines homozygous for targeted mutations at both the *Rep-3* and *mMsh2* loci and devoid of selectable markers. Thus, embryonic stem cell lines obtained in this fashion are suitable for further manipulation and analysis involving the use of selectable markers.**

The generation of mutations through homologous recombination, known as gene targeting, is a valuable tool for the functional study of genes in mammalian cells and mice (1). Gene targeting in mammalian cells is achieved when a linearized construct bearing sequences homologous to an endogenous locus recombines with its genomic target (25). Since only a small percentage of cells incorporate foreign DNA after transfection, selection for a drug resistance marker contained within the targeting vector is applied in order to obtain cells into which the construct has integrated. Southern blot analysis and/or PCR are subsequently used to identify the desired homologous recombinants from this initial pool of drug-resistant clones.

Most gene targeting strategies are designed to generate a loss-of-function (knockout) allele of the gene of interest. The most common routes toward this goal involve the use of replacement or insertion vectors (4) to disrupt the coding regions of genes. In the case of replacement vectors, selectable markers are substituted for endogenous sequences, typically resulting in the partial or complete deletion of the transcribed portions of a gene. In the case of insertion vectors, the entire targeting construct, including a selectable marker and plasmid backbone, usually integrates into the appropriate gene (3) and duplicates portions of the target locus.

Two techniques, "hit and run" (10) and "double replacement" (24), make use of a second round of homologous recombination and negative selection to generate alleles with subtle mutations which are free of selectable markers. These two methods can be used to introduce very minor changes, such as single base pair substitutions, into mammalian genes, allowing modified proteins with novel or altered functions to be generated.

The most widely used forms of gene targeting, simple replacement or insertion events, commonly result in the permanent introduction of positive selectable markers into the mammalian genome. There are instances, however, when the irreversible introduction of selectable markers as a result of gene targeting may be undesirable. First, the relatively small repertoire of positive selectable markers available for use in mammalian cells limits the number of mutations that can be generated in a single cell (5). Second, the disruption of both alleles of an autosomal gene usually requires the construction of two different targeting constructs bearing different selectable markers. Third, markers introduced by gene targeting may prevent the use of certain selection-based assays to study the mutants generated. Finally, the exogenous promoter and enhancer elements required for the expression of these selectable markers have the potential to interfere with endogenous regulatory elements present in the vicinity of the targeted mutation. Therefore, it may sometimes be advantageous to remove the selectable markers after gene targeting. Although existing methodologies such as hit and run and double replacement allow for the generation of marker-free targeted alleles, the removal of selectable markers by these techniques relies on a second, relatively inefficient round of homologous recombination.

The CRE protein of bacteriophage P1 catalyzes the conservative, site-specific recombination of *loxP* sites in mammalian cells (21). This protein has proven to be a useful tool for the manipulation of the mammalian genome, both in vitro (9, 17, 23) and in vivo (8). In this study, we describe the CRE-*loxP*-mediated, selectable excision of exogenous markers after gene targeting to generate a small insertional mutation in exon 2 of *Rep-3*, a gene homologous to the *mutS* family of DNA mismatch repair genes (14). The mutation lies within 2 kb of a bidirectional promoter shared by *Rep-3* and the *Dhfr* gene (13). With the same construct, a second round of targeting and excision was carried out to generate embryonic stem (ES) cell clones homozygous for the mutation and devoid of exogenous selectable markers. The absence of selectable markers in these ES cell lines allowed for the subsequent targeting and excision of both alleles of the mouse homolog of *Msh2* (7, 12), another

* Corresponding author. Mailing address: Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-3533. Fax: (713) 798-8142. Electronic mail address: abradley@bcm.tmc.edu.

member of the *mutS* family of genes. Thus, ES cell lines homozygous for targeted mutations at both loci and devoid of exogenous selectable markers were generated.

## MATERIALS AND METHODS

**Library screening. (i) *Rep-3*.** A 5′ primer (GGGCGGGTTCTGTCTG) complementary to sequences in the first intron of *Rep-3* and a 3′ primer (CTTTG CTTTGCTTTGA) complementary to exon 1b were used in a PCR to amplify a 624-bp genomic fragment from mouse 129Sv/Ev DNA (94°C for 1 min, 58°C for 2 min, and 72°C for 1 min for 40 cycles). The amplified product was cloned into pGEM-T (Promega) and sequenced by standard methods for verification. It was then radiolabeled (RandomPrime; Pharmacia) and used as a probe to screen a mouse 129Sv/Ev genomic library as described previously (19).

**(ii) *mMsh2*.** Degenerate PCR primers complementary to highly conserved regions of *mutS* genes were used to amplify a 263-bp fragment of the *mMsh2* cDNA, with 1 μg of reverse-transcribed (Superscript RT II first-strand cDNA synthesis kit; Gibco-BRL) total mouse embryonic (16.5-day) RNA being used as the template. Primer sequences are as follows: 5′ oligonucleotide mixture, AT(A/T/C)AC(T/G/C)GG(A/T/G/C)CC(A/G/C)AA(T/C)ATGGG, and 3′ oligonucleotide mixture, GT(A/T/C)CC(A/T)C(T/G)(T/G/C)CC(A/T/G/C)T(A/T/G/C)(T/C)TC(A/G)TC. PCR conditions were 94°C for 1 min, 62°C for 1.5 min, and 72°C for 30 s for 35 cycles. The 263-bp amplification product was cloned into pGEM-T (Promega) and sequenced by standard methods. This cloned fragment was then radiolabeled (RandomPrime; Pharmacia) and used as a probe to screen a mouse 129Sv/Ev genomic library as described previously (19).

**Vector construction.** pNTL is composed of a pBluescript SK (Stratagene) backbone containing the PGKneo$^c$bpa (28) and MC1tk (15) cassettes flanked by *loxP* sites, which were obtained from pBS64 (22) in the same relative orientation.

To generate the *Rep-3* targeting construct, pRep3ko, a 6.2-kb *Xba*I genomic fragment containing exon 2 of *Rep-3*, was inserted into the *Xba*I site of pUC18 (27). An adapter oligonucleotide pair containing *Bgl*II, *Bst*XI, and *Xho*I sites and translational termination codons in all reading frames was then inserted into the *Apa*I site of exon 2 of *Rep-3*. The pNTL cassette was then inserted into the *Bst*XI and *Xho*I sites.

To generate the *mMsh2* targeting vector pMsh2ko, an 8.5-kb *Hin*dIII-*Kpn*I genomic fragment was cloned into the pUC18 (27) polylinker region. An internal *Spe*I-*Xho*I 2-kb fragment containing two exons of the *mMsh2* gene was then excised and substituted with a *Sal*I-*Spe*I fragment containing the pNTL cassette.

**Cell culture, electroporation, selection, and analysis of targeted clones.** The procedures for ES cell culture, electroporation, drug selection, and Southern blot analysis of targeted clones have been described previously (16). Briefly, pRep3ko and pMsh2ko were prepared by alkaline lysis and purified from CsCl density gradients. They were then linearized outside the region of homology, with *Sal*I and *Kpn*I for pRep3ko and pMsh2ko, respectively, and purified by phenol-chloroform extraction and ethanol precipitation. A total of 20 μg of the linearized construct was electroporated into AB1 (15) cells or derived subclones with a Bio-Rad Gene Pulser, and $10^7$ cells were plated onto four 90-mm-diameter SNL76/7 feeder plates (15) containing M15 medium (28). At 24 h after electroporation, M15 containing geneticin (G418 sulfate; Gibco-BRL) (180 μg of the active ingredient per ml) was added to each 90-mm-diameter plate, and the cells were maintained under selection for 11 days. G418$^r$ clones were then picked and expanded in 96-well plates. Upon passaging, half of the cells in each well were frozen in a 96-well plate and the other half were plated onto a gelatinized plate without feeders and grown for another 5 to 7 days for Southern blot analysis (18). For the identification of targeted clones at the *Rep-3* locus, DNA from G418$^r$ clones was digested with *Bgl*II and hybridized to probes A and B. Probe A is a 1.25-kb external genomic fragment located 3′ to the region of homology in pRep3ko, from the *Xba*I site to the end of an isolated phage insert. Probe B is a 1.7-kb internal fragment from a 5′ *Not*I site in the *Dhfr–Rep-3* promoter region to the *Apa*I site in exon 2 of *Rep-3*. To detect targeting at the *mMsh2* locus, DNA from G418$^r$ clones was digested with *Bam*HI and hybridized to probes 1, 2, and *tk*. Probe 1 is a 1.7-kb *Rsa*I genomic fragment external to the region of homology in pMsh2ko on its 5′ end. Probe 2 is a 0.7-kb *Kpn*I-*Bam*HI genomic fragment external to the region of homology in pMsh2ko on its 3′ end. Probe *tk* is a 1.95-kb fragment containing the *tk* coding region of the MC1tk (15) cassette.

**CRE-*loxP*-mediated marker excision.** Clones identified as targeted with the appropriate probes were thawed and expanded for 8 to 10 days in SNL76/7 feeder plates containing G418-supplemented M15 medium. Half of the cells for each clone were then frozen (16), and the remaining cells were grown in M15 for 3 days. A total of $10^7$ cells were then electroporated with either 20 μg of supercoiled pOG231, a mammalian CRE expression vector (gift from Steve O'Gorman, The Salk Institute), or Tris-EDTA (negative controls). After electroporation, the cells were plated onto a 90-mm-diameter SNL76/7 feeder plate containing M15. The cells were maintained and passaged as needed in M15 for 5 days. A serial dilution of the cells was then plated onto six-well SNL76/7 feeder plates containing M15 supplemented with 1-(-2-deoxy-2-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil (FIAU) (15). Cells were maintained under selection for 12 days, and FIAU$^r$ colonies were counted, picked, and expanded in 96-well plates for Southern blot analysis.

To determine the efficiency of CRE-mediated excision of the pNTL cassette without the use of negative selection, the same procedure was followed, except that the cells were plated at low density onto 90-mm-diameter SNL76/7 feeder plates containing M15 medium alone. After growth for 12 days, colonies were picked and expanded in 96-well plates for Southern blot analysis.

**Amplification and sequencing of the mutated *Rep-3* allele.** Two oligonucleotide primers, CAGAAGGTGACAGCAGGAAGAGG and GGGTGGTGAGA TGCTACTGAGAT, complementary to the 5′ and 3′ ends of exon 2 of *Rep-3*, respectively, were used in PCRs (94°C for 1 min, 58°C for 2 min, and 72°C for 1 min for 38 cycles). DNA from wild-type AB1 controls and the appropriate FIAU$^r$ clones was used as the template for amplification. Amplification products were cloned into pGEM-T (Promega) and sequenced by standard methods.

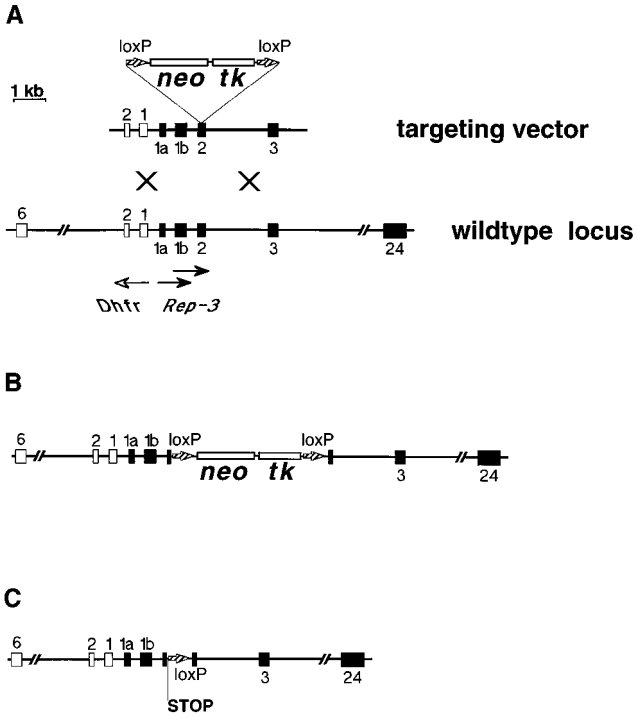


FIG. 1. Strategy for creating a marker-free insertion in exon 2 of *Rep-3*. (A) Structure of the wild-type *Dhfr–Rep-3* genomic locus and targeting vector. Numbered open and solid boxes represent *Dhfr* and *Rep-3* exons, respectively (adapted from references 13 and 14). *Dhfr* and *Rep-3* are transcribed in opposite directions from a common bidirectional promoter, as represented by the open and solid arrows, respectively. Translational start sites for REP-3 are contained within alternative exons 1a and 1b (13). (B) Predicted structure of the locus after homologous recombination. (C) Predicted structure of the locus after CRE-*loxP*-mediated excision of the markers from the targeted allele. *neo*, pGKneo$^c$bpa expression cassette; *tk*, HSV-*tk* expression cassette; loxP, *loxP* site.

## RESULTS

The strategy used to introduce a small insertional mutation into exon 2 of *Rep-3* is depicted in Fig. 1. The targeting construct pRep3ko was used to target the *Rep-3* locus in mouse ES cells. This targeting vector contains neomycin resistance (*neo*) and herpes simplex virus thymidine kinase (HSV-*tk*) expression cassettes, flanked by *loxP* sites, inserted into exon 2 of *Rep-3*. The *loxP* sites are in the same relative orientation so that the expression cassettes which lie between the *loxP* sites are excised upon CRE-mediated recombination. A series of translational termination codons present 5′ of the *loxP-neo-tk-loxP* cassette was designed to be left behind in the genome along with a single *loxP* site and polylinker sequences after CRE-mediated excision. Exon II was selected as the target for the mutation because it is the first translated exon common to all *Rep-3* mRNA transcripts (13). pRep3ko was electroporated into AB1 mouse ES cells (15), G418 selection was applied, and genomic DNA from G418$^r$ clones was screened by Southern

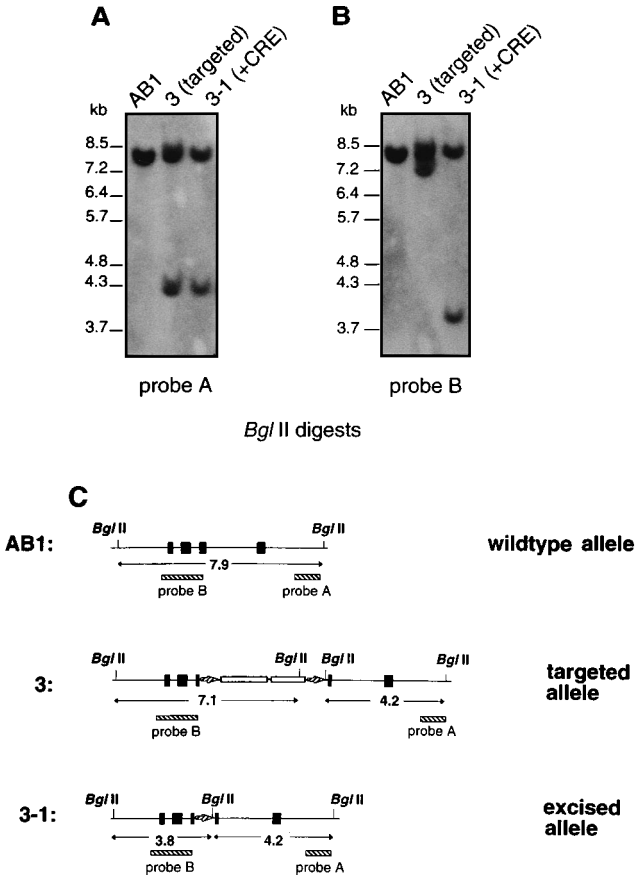


FIG. 2. Targeting and marker excision of one allele of *Rep-3*. (A and B) DNA from a targeted clone and wild-type AB1 cells was examined by Southern blot analysis. All lanes contain 10 μg of *Bgl*II-digested genomic DNA. DNA size markers are indicated on the left of each panel. The same blot was probed with probe A (A) and probe B (B). (C) Schematic representation of the wild-type and mutant alleles in AB1 cells and clones 3 and 3-1. Numbers represent fragment sizes in kilobases.

analysis to identify targeted clones with probes A and B. Probe A detected the predicted size change from the wild-type 7.9-kb fragment to a 4.2-kb *Bgl*II fragment in 5 of 346 G418$^r$ clones scored. Three of these clones (clones 2, 3, and 4) were tested with probe B, and all had the predicted 7.1-kb *Bgl*II fragment (Fig. 2).

In order to remove the selectable markers from exon 2 of *Rep-3*, confirmed targeted clones (2, 3, and 4) were transiently transfected with pOG231, a mammalian CRE expression vector, and placed in FIAU to select against HSV-*tk* expression. ES cell clones that had excised the drug resistance markers were recovered. To determine the efficiency of CRE-*loxP*-mediated excision relative to background levels of FIAU resistance (due to spontaneous mutation or the loss of the HSV-*tk* cassette), the targeted clones were mock transfected and selected in FIAU under the same conditions as those for the CRE-transfected samples. The relative number of FIAU$^r$ clones for both samples is presented in Table 1. FIAU-resistant clones from the experimental (with CRE) group were picked, expanded, and subjected to Southern analysis with probe B to confirm the structure of the excised allele. After CRE-*loxP*-mediated excision of the *neo* and HSV-*tk* cassettes, probe B should detect a predicted size change from the previous 7.1-kb allele to a new 3.8-kb allele with *Bgl*II-digested DNA (Fig. 2). The results of this analysis are also summarized in Table 1.

TABLE 1. Efficiency of CRE-*loxP*-mediated marker excision

| Targeted clone | Relative no. of FIAU$^r$ colonies | | No. of colonies positive by Southern analysis/no. scored[a] |
|---|---|---|---|
| | With CRE | Without CRE | |
| 2 | 474 | 1 | 17/17 |
| 3 | 87 | 1 | 33/33 |
| 4 | 114 | 1 | 4/4 |

[a] *Bgl*II-digested genomic DNA from a subset of clones from the class "with CRE" was examined by Southern analysis with probe B.

To determine the efficiency of CRE-mediated excision of the selectable markers without the use of FIAU negative selection, clone 3 was transiently transfected with the mammalian CRE expression vector pOG231 and plated at clonal density without selection. Colonies were picked and screened for the CRE-mediated excision event with probe B on *Bgl*II-digested DNA. Of 378 independent clones screened by Southern analysis, 60 (16%) had undergone the predicted size change from 7.1 to 3.8 kb (data not shown).

To verify the structure of the mutation, *Rep-3* exon 2 sequences were amplified from CRE-induced FIAU$^r$ clones and wild-type AB1 controls by PCR. An additional amplification product larger than the 112-bp wild-type allele was observed in PCRs with FIAU$^r$ clones but not control AB1 cells (data not shown). For one clone (clone 3-1), this larger fragment was cloned into pGEM-T (Promega) and sequenced. Sequencing revealed that the predicted 181-bp insertion had been generated in exon 2 of *Rep-3*. The insertion contains the expected single *loxP* site, polylinker sequences, and translational termination codons in all reading frames (Fig. 3).

The clones with the 181-bp insertion are now devoid of selectable markers; thus, they are suitable substrates for the use of the same targeting vector to modify the other allele of *Rep-3*. To target the second allele of *Rep-3*, pRep3ko was electroporated into clone 3-1 and G418$^r$ colonies were screened for the targeting event with external probe A. Although pRep3ko is expected to target either the wild-type or the previously targeted allele with similar frequencies, retargeting of the previously targeted allele by pRep3ko cannot be scored when external probe A is used. Four of 356 G418$^r$ clones were positive (clones 3-1-A through 3-1-D) and exhibited the predicted size change from a 7.9- to a 4.2-kb *Bgl*II

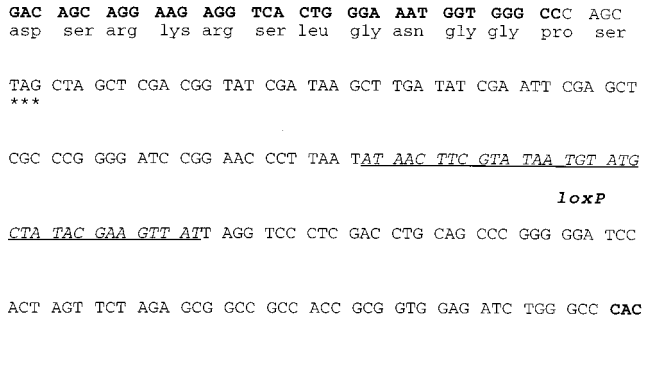


FIG. 3. Nucleotide sequence of the insertional mutation generated in clone 3-1. *Rep-3* exon 2 sequences flanking the insertion are shown in boldface. The single *loxP* site is shown underlined. The first in-frame translational termination codon is denoted with three asterisks.

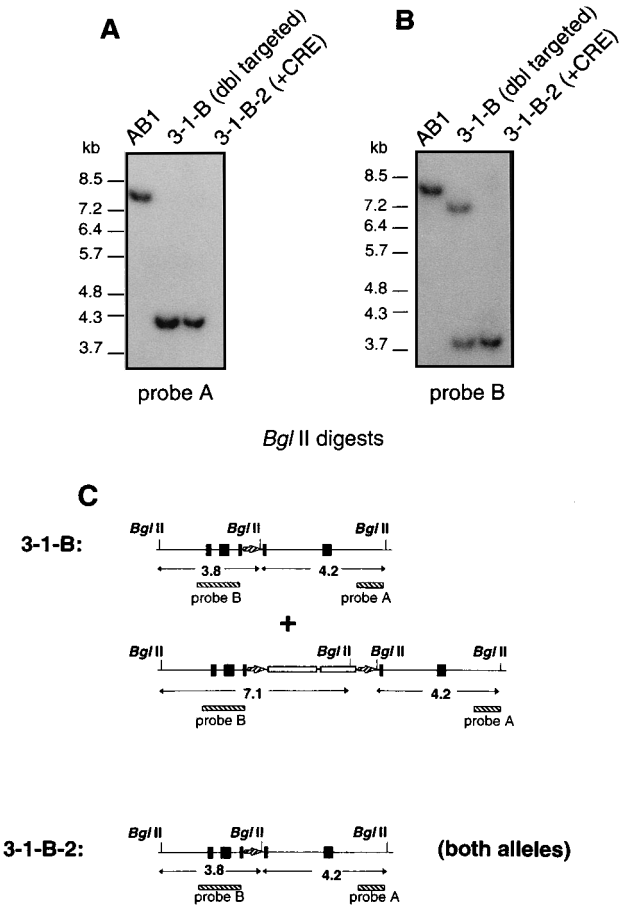


FIG. 4. Targeting and marker excision of the remaining wild-type allele of *Rep-3*. (A and B) DNA from a double-targeted clone and wild-type AB1 cells was examined by Southern blot analysis. All lanes contain 7 μg of *Bgl*II-digested genomic DNA. DNA size markers are indicated on the left of each panel. The same blot was probed with probe A (A) and probe B (B). (C) Schematic representation of the mutant alleles in clones 3-1-B and 3-1-B-2. Numbers represent fragment sizes in kilobases.

fragment for the remaining wild-type allele of *Rep-3* with probe A. Two of these clones (3-1-B and 3-1-C) were tested with probe B. Both clones exhibited the predicted size change from 7.9 to 7.1 kb (Fig. 4). Clones 3-1-B and 3-1-C were subjected to CRE-*loxP*-mediated excision of the drug resistance markers as before by applying FIAU selection after transient transfection of the CRE expression vector pOG231. A subset of FIAU$^r$ clones was examined by Southern analysis with probe B, which confirmed the size change from 7.1 to 3.8 kb after the excision of the selectable markers (Fig. 4).

The random integration of the pOG231 CRE expression plasmid into the ES cell genome after transient transfection could result in mutation events at the site of integration or in the constitutive expression of the CRE protein, which might interfere with subsequent attempts at targeting with a vector bearing *loxP* sites. In order to assess the frequency of pOG231 integration into the genome after transfection, a DNA fragment encompassing the *cre* coding regions in pOG231 was used as a probe to screen DNA from clones in which the CRE-mediated excision event had been confirmed by Southern analysis. As positive controls, DNA from clones which had been transfected with a mixture of linearized pOG231 and a neomycin resistance vector and grown under G418 selection was included in the screen. Of 22 clones screened, none showed hybridization to the *cre* probe. The probe did hybridize to DNA from positive controls (data not shown).

The absence of selectable markers in the ES cell lines homozygous for the targeted insertion at the *Rep-3* locus allowed us to use the same pNTL cassette to introduce a mutation at the *mMsh2* locus, another member of the *mutS* family of genes (7, 12). Figure 5 shows the strategy used for generating a 2-kb marker-free deletion in the *mMsh2* genomic locus. This 2-kb fragment encompasses much of the most conserved region among *mutS* genes. Sequencing of the entire 2-kb fragment revealed the presence of two exons with sizes of 205 and 248 bp, respectively, that were separated by a 0.5-kb intron. These exons show 100% sequence identity with the published *mMsh2* cDNA sequence (26) and correspond to exons 13 and 14 of the published human *Msh2* genomic locus (11) (data not shown).

The *Rep-3*-deficient clone 3-1-B-2 (Fig. 4) was transfected with pMsh2ko, and G418$^r$ clones were screened by Southern analysis with probe 2 on *Bam*HI-digested DNA. Two of 93

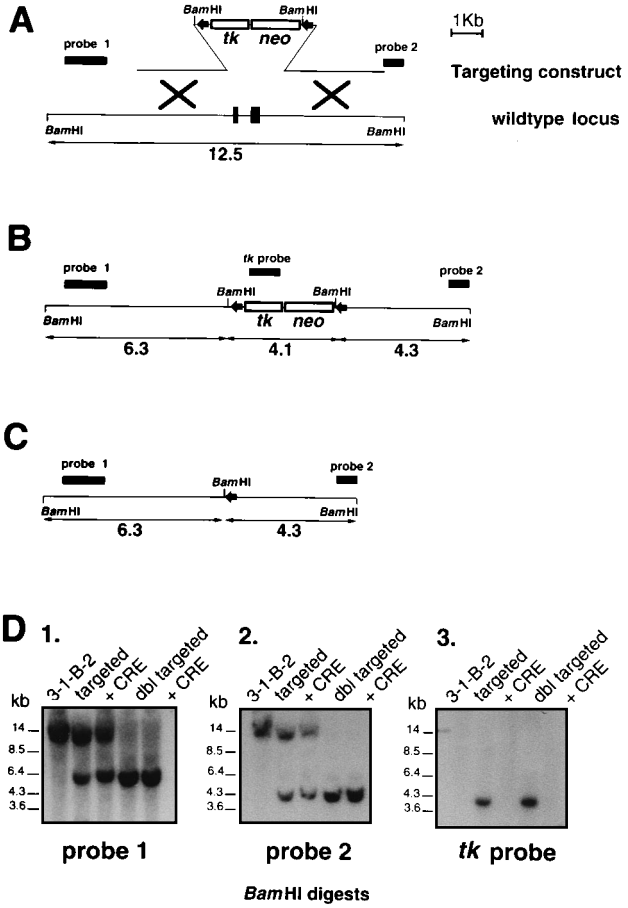


FIG. 5. Strategy for creating a marker-free deletion at the *mMsh2* locus. (A) Structure of the wild-type *mMsh2* genomic locus and targeting vector. Gray boxes represent the 205- and 248-bp exons described in the text (see Results). Gray arrows represent *loxP* sites. Numbers represent fragment sizes in kilobases. *neo*, pGKneo$^c$bpa expression cassette; *tk*, HSV-*tk* expression cassette. (B) Predicted structure of the locus after homologous recombination. (C) Predicted structure of the locus after CRE-*loxP*-mediated excision of the markers from the targeted allele. (D) Southern blot analysis of DNA from clone 3-1-B-2, clone 3-1-B-2 with a targeted *mMsh2* allele, the same clone after CRE-mediated marker excision, the same clone after targeting of the second *mMsh2* allele, and the double-targeted clone after CRE-mediated excision. *Bam*HI-digested DNA (7 μg) was loaded in each lane.

clones screened showed the predicted size change from 12.5 to 4.3 kb for one allele of *mMsh2*. Both of these clones showed the predicted size change from 12.5 to 6.3 kb with probe 1 and the expected 4.1-kb fragment when hybridized to the *tk* probe (Fig. 5). The selectable markers were then removed by CRE-mediated excision after transient transfection with pOG231 and FIAU selection as before. To verify the excision of the markers, Southern analysis of DNA from FIAU$^r$ clones was carried out with the *tk* probe. Two clones were analyzed, and both showed an absence of hybridization to the *tk* probe (Fig. 5). To target the remaining allele of *mMsh2*, one such FIAU$^r$ clone was again transfected with pMsh2ko and G418$^r$ clones were screened by Southern analysis with probe 2 on *Bam*HI-digested DNA. As with pRep3ko, pMsh2ko is expected to recombine with either the previously targeted allele or the remaining wild-type allele. Retargeting of the previously targeted allele cannot, however, be scored with either probe 1 or 2. Three of 95 clones screened had undergone the predicted size changes when hybridized to probes 1 and 2 for the remaining wild-type *mMsh2* allele. All three clones showed the predicted 4.1-kb band when hybridized to the *tk* probe (Fig. 5). Two of these clones were then subjected to FIAU selection after transient transfection of the CRE expression vector to remove the selectable markers. Two FIAU$^r$ clones (one for each clone) were tested by Southern analysis with the *tk* probe to verify the excision of the markers. Both showed an absence of hybridization to the *tk* probe (Fig. 5).

## DISCUSSION

The CRE-*loxP* system has been shown to be an efficient tool for the generation of defined genomic rearrangements, such as small deletions (9) in mouse ES cells. CRE also mediates other chromosomal changes involving larger fragments, but at a much lower frequency (17, 23). CRE-*loxP*-mediated excision of selectable markers after gene targeting has been proposed as a method for the recycling of markers in yeast cells (20). In this study, we have described the combined use of homologous and CRE-*loxP*-mediated recombination to generate mouse ES cell lines that are homozygous for targeted mutations at both the *Rep-3* and *mMsh2* loci and which contain no exogenous selectable markers. The absence of drug resistance markers in these cell lines offers several advantages over cell lines generated with standard protocols. First, the lack of markers renders these cell lines suitable for additional targeting events at different loci and allows for the use of selection-based assays to study the cellular roles and possible functional interactions of REP-3 and mMSH2. Furthermore, we avoided additional vector construction by targeting both alleles of *Rep-3* and *mMsh2* with a single targeting construct in each case. Finally, in the case of *Rep-3*, we reduced the risk of interfering with the normal expression of the nearby *Dhfr* gene (13) by generating a mutation devoid of exogenous regulatory elements. A similar strategy using a different recombinase system has recently been reported for the deletion of a putative regulatory element at the murine β-globin locus (6).

We observed a low ratio of background FIAU$^r$ colonies relative to CRE-induced FIAU$^r$ clones (1:225 on average). Moreover, 100% of the CRE-induced FIAU$^r$ clones tested by Southern analysis had undergone the predicted genomic rearrangement. Thus, from a practical standpoint, it is unnecessary to isolate and characterize individual ES cell clones after CRE-*loxP*-mediated marker excision from the first targeted allele, making it possible to handle CRE-induced FIAU$^r$ clones as a population for the purpose of targeting the second allele. When ES cell-derived mice are generated (2), it may also be prudent to pool and grow multiple CRE-induced FIAU$^r$ colonies together as a population in order to avoid any problems associated with individual ES cell clones.

We observed a relatively high percentage (16%) of clones which had undergone the CRE-mediated marker excision event in the absence of negative selection after transient transfection of the CRE expression vector. Although this frequency is high, it is possible that the efficiency of CRE-mediated recombination of the *loxP* sites varies among different loci because of such structural factors as chromatin structure or accessibility. We believe that negative selection for the CRE-mediated excision event is desirable, since it eliminates the necessity to subclone and screen CRE-transfected clones, which expedites experiments and avoids potential problems associated with individual clones.

We observed no integration of the CRE expression vector into the genome after transient transfection in 22 clones analyzed. Although such an event might lead to the constitutive expression of the CRE protein, which might interfere with subsequent attempts at the use of the CRE-*loxP* system in the same clone, it is likely to be exceedingly rare, since stable integration occurs at a frequency of fewer than $10^{-3}$ per transfected cell.

The expression of CRE in cell lines which contain a number of *loxP* sites has the potential to induce the recombination of homologs and could translocate chromosomes when the *loxP* sites are inserted into two or more genes, such as *Rep-3* and *mMsh2*, which map to different chromosomes. However, we have documented that the recombination of *loxP* sites in *trans* (homologous chromosomes) is at least 2 to 3 orders of magnitude less efficient than when in *cis* (on the same chromosome) (17). Moreover, recombination of homologs is functionally neutral in an ES cell line derived from an inbred mouse. CRE-induced translocations have been documented (23), but the frequency of inducing these events is at least 6 orders of magnitude less than that for the simple excision events described here. Thus, it is unlikely that the expression of CRE will induce any unwanted chromosomal alterations at a significant frequency compared with those of the selected simple excision event.

In principle, CRE-*loxP*-mediated excision of selectable markers after gene targeting allows for the generation of an unlimited number of targeted mutations in a single mammalian cell line. Mutant cell lines generated in this way are suitable for subsequent genetic analysis with selection-based assay systems. This system may prove to be particularly useful for the study of mammalian genes whose functions can be assayed at the cellular level, such as DNA repair genes. Cell lines homozygous for mutations in multiple genes may prove useful for the investigation of possible functional relationships among their protein products.

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