MOLECULAR AND CELLULAR BIOLOGY, May 1992, p. 2391–2395
0270-7306/92/052391-05$02.00/0
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Vol. 12, No. 5

Production of Homozygous Mutant ES Cells with a Single Targeting Construct

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Received 26 December 1991/Accepted 20 February 1992

We have developed a simple method for producing embryonic stem (ES) cell lines whereby both alleles have been inactivated by homologous recombination and which requires a single targeting construct. Four different ES cell lines were created that were heterozygous for genes encoding two guanine nucleotide-binding protein subunits, α_{i2} and α_{i3}, T-cell receptor α, and β-cardiac myosin heavy chain. When these heterozygous cells were grown in high concentrations of G418, many of the surviving cells were homozygous for the targeted allele and contained two copies of the G418 resistance gene. This scheme provides an easy method for obtaining homozygous mutationally altered cells, i.e., double knockouts, and should be generally applicable to other genes and to cell lines other than ES cells. This method should also enable the production of cell lines in which more than one gene have had both alleles disrupted. These mutant cells should provide useful tools for defining the role of particular genes in cell culture.

With the discovery that gene sequences in mammalian cells could be targeted by homologous recombination, new methods for constructing mutationally altered cells that lack particular genes have become available. Over the past several years, a number of groups have demonstrated that heterozygous cells in which one of two alleles has been inactivated can be constructed (4, 7, 8, 14, 16, 18, 19).

Production of null mutants by using homologous recombination (in which two copies of the gene have been inactivated) has recently been accomplished in embryonic stem (ES) cells (9, 15) and other types of cells (3). Such mutants should allow the investigation of questions more easily addressed in cell lines rather than in whole animals or tissues. The methods used involved sequential targeting by two separate constructs using two different selectable markers. Here, we describe an improved method for obtaining such null mutant cells. Homozygous cells have been known to be spontaneously produced from heterozygous cultured cells by a variety of mechanisms (2, 10, 12, 13). We demonstrate that homozygous cells are routinely produced after initially targeting a single allele and have developed a scheme for the selection of these double knockouts.

MATERIALS AND METHODS

Construction of targeting vectors. In each case, the phosphoglycerate kinase (PGK)-thymidine kinase gene was inserted outside the regions of homology. The production of the α_{i2} targeting construct has been described previously (9). The α_{i3} gene clone was isolated as described for the α_{i2} gene. A *Hin*dIII-*Sal*I fragment was subcloned into Bluescript SK (+) (Stratagene). The construct contained the PGK-*neo* (neomycin resistance) gene interrupting exon 1 at an *Nco*I site at the translational start ATG. A 5.6-kb fragment of the murine T-cell receptor α (TCR-α) gene containing all four constant-region exons was subcloned from genomic clone λ*1.2 (6) into Bluescript SK II (+). The fragment extends from an artificial *Eco*RI site, 5′ of the *Xba*I site, to the *Xho*I site (Fig. 2). The *Eco*RI site between exons 3 and 4 was deleted by digestion with *Eco*RI, blunting with Klenow enzyme, and subsequent ligation. The first constant-region exon was interrupted at a unique *Eco*RV site by the insertion of PGK-*neo*. A 7.5-kb (*Sal*I-*Eco*RV) portion of the *Myhc-b* (β-myosin heavy chain) gene extending from approximately exons 10 to 24 was cloned from a BALB/c genomic library (Clontech); identity was confirmed by sequencing. A targeting construct was made by interrupting the gene at a unique *Nde*I site in exon 14.

ES cell culture and G418 selection for null mutants. ES cells (line CCE, obtained from E. Robertson, Columbia University, or CC1.2, obtained from A. Bradley, Baylor University) were cultured, transfected, and screened for homologous recombination as previously described (9). Several cell lines identified as heterozygous for homologous recombination were expanded for 14 to 28 days. Cell lines were then plated, cultured for 24 h, and then selected at various concentrations of G418 (up to 2.0 mg/ml). DNA from the surviving clones was analyzed by Southern blot for absence of the endogenous gene.

G418 resistance of ES cell clones. Wild-type CCE cells or one heterozygous (containing one copy of *neo*) or two homozygous (containing two copies of *neo*) ES cell lines were plated at 10^4 cells per 150-mm plate. One day later, 0, 0.1, 0.2, 0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 mg of G418 per ml was added to the culture media. After 2 weeks, the number of surviving colonies was determined. Results were normalized to values for the plate containing no G418.

Second cloning and selection. Four individual heterozygous subclones were isolated by plating trypsin-dispersed 17E10 cells at a high dilution. Each of these subclones and the original 17E10 clone were plated at 5×10^5 cells per 100-mm plate and selected at high G418 levels, and the surviving clones were analyzed as described above.

Estimation of rate of loss of heterozygosity. The rate was

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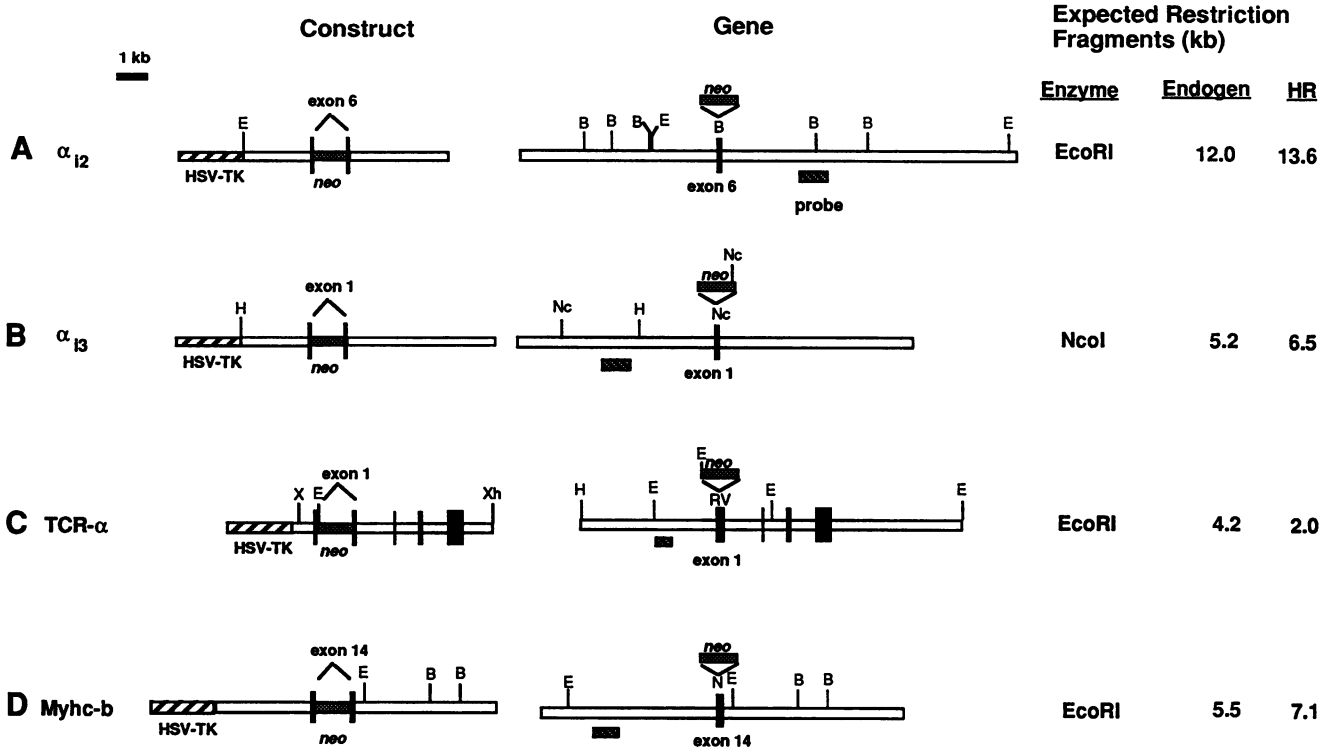

FIG. 1. Maps of the targeted genes and constructs for homologous recombination. Restriction enzyme sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; Xh, *Xho*I; Nc, *Nco*I; RV, EcoRV; N, *Nde*I. The solid vertical bars indicate the exons interrupted by the *neo* gene. The expected sizes of restriction fragments detected by the probes indicated for each endogenous and targeted gene are shown. Abbreviations: Endogen, endogenous gene; HR, homologous recombination; HSV, herpes simplex virus; TK, thymidine kinase.

calculated from the number of generations since recloning (assuming a generation time of 14.2 h) (9) and the fraction of homozygotes {(average number of colonies)/[(number of cells plated) × (plating efficiency)]} according to the equation rate = (fraction of homozygotes)/(number of generations since cloning). Cells were cultured for 16 days (27 generations) after plating at a high dilution.

Southern and Northern (RNA) analyses. DNA was fractionated on 1% agarose gels, transferred to GeneScreen, and hybridized as described elsewhere (1). RNA from each cell line was fractionated on a formaldehyde-agarose gel (1%), transferred to GeneScreen, hybridized to a 1.4-kb genomic fragment containing exon 4 from the α_{i2} gene (probe A; Fig. 1) or with a *Pst*I-*Pst*I fragment from the coding region of the *neo* gene as described elsewhere (1), and washed two times at 70°C in 0.2× SSC (1× SSC is 0.015 M NaCl plus 0.015 M sodium citrate) for 20 min each time.

RESULTS

Four separate genes, G-protein subunits α_{i2} and α_{i3}, TCR-α, and *Myhc-b*, were targeted by using positive-negative selection (8). In each case, we made a targeting construct containing the *neo* gene driven by the promoter region of the PGK gene. The PGK-*neo* sequence interrupted an exon creating a detectable change in the gene's restriction enzyme digest pattern (Fig. 1). Transfection of ES cells, selection, and identification of heterozygous homologous recombinants were performed as previously described (9).

We have previously described the production of ES cells containing an inactivated gene of the G-protein subunit α_{i2} by homologous recombination (construct shown in Fig. 1A). These heterozygous cells were used to produce an ES cell line lacking a functional α_{i2} gene by homologous recombination with a *hyg* gene-containing construct (9). During our analysis of putative α_{i2}-deficient cells, we found clones that had not undergone homologous recombination with the *hyg* construct yet lacked the endogenous α_{i2} gene. Southern blot analyses of the DNA from these colonies are shown in Fig. 2A (designated −/−). These colonies preexisted the transfection with the α_{i2}-*hyg* construct, since the *hyg* gene was randomly integrated into the genome (data not shown). The number of alleles in each cell was estimated by Southern blot analysis with a probe from the α_{i2} gene and by using hybridization to a 21-hydroxylase probe as a control for the amount of DNA loaded in each lane (Fig. 1A). The results, summarized in the legend to Fig. 2A, show that the clones are diploid at the locus. This conclusion was supported by the observation that the cells had a normal karyotype with 40 chromosomes (data not shown). These data suggested that ES cells, like other cultured cells, undergo a loss of heterozygosity.

Expression of the α_{i2} gene and *neo* was characterized by Northern analysis. There was no detectable α_{i2} mRNA (Fig. 3A, lanes 3 and 4), confirming inactivation of both copies of the α_{i2} gene. The Northern analysis also indicated that these colonies expressed *neo* to a greater degree, as might be expected for a cell line containing two *neo* copies per diploid instead of one (Fig. 3A, lanes 7 and 8). The finding that cells bearing two copies of *neo* express more *neo* mRNA suggested that homozygous colonies would be more resistant than heterozygous cells to G418 (11). A higher degree of drug

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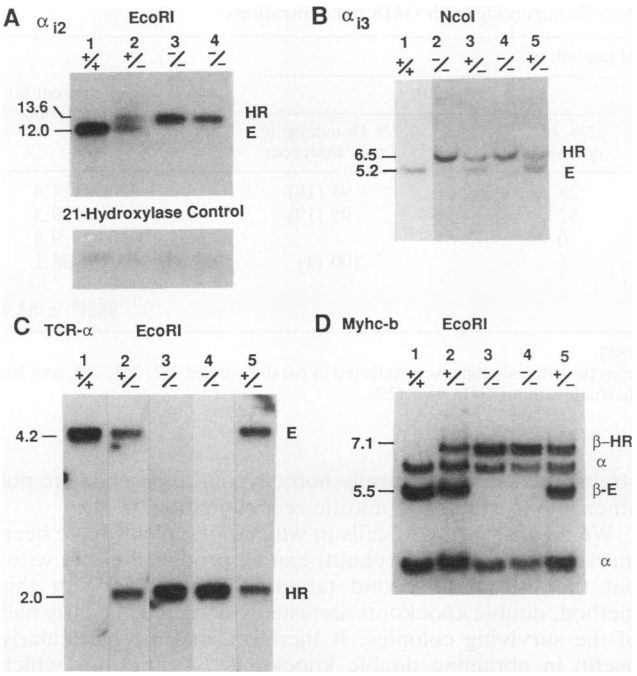

FIG. 2. Southern blot analysis of DNA from clones homozygous for a *neo* interruption of the gene. In each case, results for wild-type (+/+) and heterozygous (+/−) or independent double-knockout (−/−) ES cell lines are shown. The restriction enzymes used and the sizes (in kilobases) of the bands are indicated. The bands expected from the endogenous gene (E) and resulting from homologous recombination (HR) are also shown. (A) α_{i2} gene. The homozygous clones are those previously isolated (9). Included are results from reprobing the same blots for the α_{i2} gene with a probe for the 21-hydroxylase gene to control for DNA loading. Densitometry in three separate experiments (standardized by using the wild-type CCE band as 2.0 copies) showed a relative copy number of 1.92 ± 0.34. (B) α_{i3} gene; (C) TCR-α gene; (D) *Myhc-b* gene. This β-cardiac myosin probe cross-reacts with the *Myhc-a* (α-cardiac myosin heavy chain) gene, resulting in the two bands (labeled α) present in each lane.

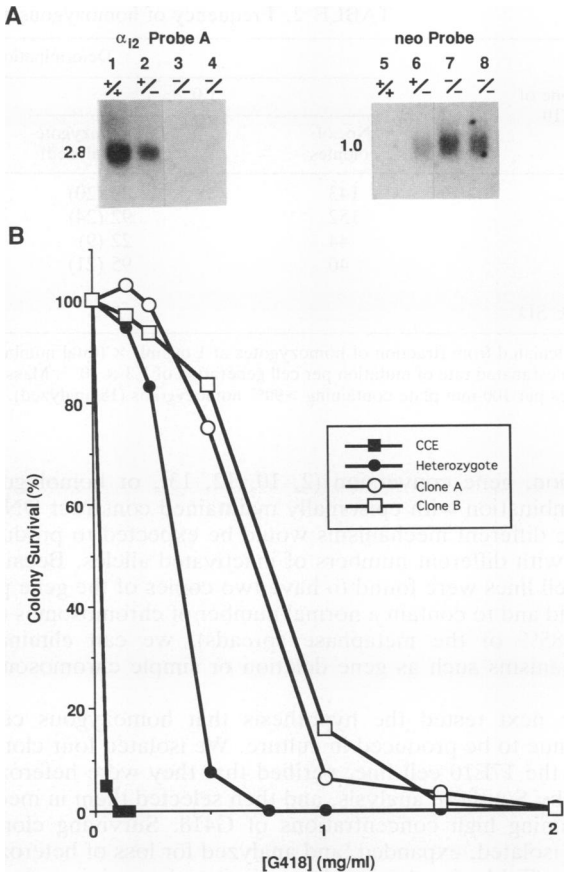

FIG. 3. (A) Northern analysis of total RNA isolated from wild-type (+/+), α_{i2}-heterozygous (+/−), or two independent α_{i2}-null (−/−) ES cell lines. RNA (20 μg) hybridized to an α_{i2} probe A (Fig. 1) or *neo* probe. (B) G418 resistance of ES cell clones, wild-type CCE cells, heterozygous cells bearing one *neo* copy, and two independent clones (A and B in Fig. 1) bearing two *neo* copies.

resistance was confirmed by culturing in higher concentrations of G418 (Fig. 3B). These results suggested that double knockouts can be obtained by selection in high G418 concentrations.

To test this hypothesis, seven separate cell lines, each heterozygous at one of the four targeted loci, were cultured in high concentrations of G418 (from 1.0 to 2.0 mg/ml). Surviving clones were isolated and expanded, and their DNA was analyzed by Southern blotting (Fig. 2B to D). In each case, many clones which lacked the endogenous gene were obtained (summarized in Table 1).

These cells could have arisen by a number of mechanisms, including chromosomal loss, gene deletion, mitotic recom-

TABLE 1. Loss of heterozygosity in ES cell lines

Gene (parent line)	Cell line	No. of cells plated[a]	Determination at [G418] (mg/ml) of: 1.0–1.5 No. of colonies	1.0–1.5 % Homozygote (no. analyzed)	2.0 No. of colonies	2.0 % Homozygote (no. analyzed)
α_{i2} (CCE)	17E10	2×10^4	23	100 (21)	2	0 (2)
	18D3	2×10^4	175	12 (26)	40	ND[b]
α_{i3} (CC1.2)	32-32	1×10^5	100	53 (17)	17	88 (17)
	32-28	1×10^5	27	4 (28)	0	
TCR-α (CCE)	1A4	5×10^5	37	43 (23)	9	44 (9)
Myhc-b (CC1.2)	6-6	5×10^5			54	24 (54)
	6-22	5×10^5	76	7 (76)	2	50 (2)

[a] Efficiency of plating, 40 to 80%.
[b] ND, not determined.

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TABLE 2. Frequency of homozygous mutant ES cells surviving high G418 concentrations

Subclone of 17E10	Determination at [G418] (mg/ml) of:				Apparent no. of colonies[a]
	1.0		2.0		
	No. of colonies	% Homozygote (no. analyzed)	No. of colonies	% Homozygote (no. analyzed)	
1	143	80 (20)	28	94 (18)	114.4
2	152	92 (24)	52	95 (19)	139.3
3	44	22 (9)	0		9.8
4	40	95 (21)	5	100 (4)	38.1
Avg ± SD					75.4[b] ± 61.4

[a] Calculated from (fraction of homozygotes at 1 mg/ml) × (total number of colonies).
[b] An estimated rate of mutation per cell generation of 1.3×10^{-5}. Mass cultures from the original clone were selected in parallel, giving 241, 261, 228, and 262 colonies per 100-mm plate containing >90% homozygotes (18 analyzed). Plating efficiency without G418 was 42%.

bination, gene conversion (2, 10, 12, 13), or homologous recombination with episomally maintained construct DNA. These different mechanisms would be expected to produce cells with different numbers of inactivated alleles. Because the cell lines were found to have two copies of the gene per diploid and to contain a normal number of chromosomes (40 in >85% of the metaphase spreads), we can eliminate mechanisms such as gene deletion or simple chromosomal loss.

We next tested the hypothesis that homozygous cells continue to be produced in culture. We isolated four clones from the 17E10 cell line, verified that they were heterozygous by Southern analysis, and then selected them in media containing high concentrations of G418. Surviving clones were isolated, expanded, and analyzed for loss of heterozygosity (Table 2). Again, the majority of surviving clones were homozygous knockouts. From these data, the mutation rate was estimated to be 1.3×10^{-5} per cell generation.

DISCUSSION

This rate of production of homozygous mutants is similar to that observed for other cultured somatic cells (2, 10, 12, 13). However, since approximately 10% of the homozygous cells will survive these levels of G418 (Fig. 3B), this estimated rate is probably 10-fold less than the actual rate. The preferred mechanism appears to differ from one cell line to another. Wasmuth and Hall found that chromosomal loss followed by chromosomal duplication is the most common mechanism by which heterozygous alleles of CHO cells became homozygous, although mitotic recombination does occur at a lower frequency (17). Campbell and Worton (2) found that recessive phenotypes could be expressed secondary to simple chromosomal loss or chromosomal loss followed by chromosomal duplication. They did not find evidence for mitotic recombination. Human cell lines have also been shown to produce homozygous loci from originally heterozygous sites at a low frequency, although the mutational mechanism is not defined (5). In murine lymphoid cell lines, Rajan and coworkers found homozygous cells produced mainly by mitotic recombination; the rate of mitotic recombination was estimated to be 10^{-5} to 10^{-6} per generation by fluctuation analysis (10, 12, 13). We cannot currently distinguish among the remaining possible mechanisms for production of the homozygous cells. Further studies to determine whether mitotic recombination is occurring will require ES cells which are heterozygous at a number of loci. Since currently available ES cells are derived from inbred strains, all loci are naturally homozygous and hence are not amenable to studies of mitotic recombination.

We have shown that cells in which both alleles have been inactivated (double knockout) can be produced easily without the use of a second targeting construct. With this method, double knockouts are usually detected in nearly half of the surviving colonies. It therefore may be particularly useful in obtaining double knockouts of genes for which homologous recombination occurs at low rates. Another advantage of this method is that it does not rely on the phenotype of the gene and so should be applicable to any gene whose product is not required for cell viability. We demonstrate the utility of this method to produce cell lines lacking both expressed (α_{i2} and α_{i3}) and unexpressed (TCR-α and *Myhc-b*) genes in seven separately produced clones. These cells are resistant only to neomycin and its analogs, thus preserving other drug selections, such as for hygromycin B, for further genetic manipulations of the cells such as the knockout of other genes. We also expect that this method will be applicable to other cell lines and will aid investigations in cell biology and biochemistry. Since the normal number of chromosomes of the ES cells is maintained, this method should be applicable to the study of gene function in development. Cells homozygous for the α_{i2}, α_{i3}, and *Myhc-b* genes have been differentiated in vitro and are indistinguishable from wild-type cells. Mutant cells like these should provide useful tools for defining the role of particular genes in differentiated or undifferentiated ES cells.

ACKNOWLEDGMENTS

We thank W. Strauss, D. Milstone, and E. J. Neer for helpful suggestions and discussions.

This work was supported by the Howard Hughes Medical Institute. A.A.T.G.L. was supported by the Canadian Heart and Stroke Foundation and the American Heart Association, Massachusetts affiliate.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1988. Current protocols in molecular biology. John Wiley & Sons, New York.
2. **Campbell, C. E., and R. G. Worton.** 1981. Segregation of recessive phenotypes in somatic cell hybrids: role of mitotic recombination, gene inactivation, and chromosome nondisjunction. Mol. Cell. Biol. **1**:336–346.
3. **Cruz, A., C. M. Coburn, and S. M. Beverley.** 1991. Double targeted gene replacement for creating null mutants. Proc. Natl. Acad. Sci. USA **88**:7170–7174.
4. **Doetschman, T., R. G. Gregg, N. Maeda, M. L. Hooper, D. W.**

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Melton, S. Thompson, and O. Smithies. 1987. Targeted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature (London) **330:**576–578.
5. **Groden, J., Y. Nakamura, and J. German.** 1990. Molecular evidence that homologous recombination occurs in proliferating human somatic cells. Proc. Natl. Acad. Sci. USA **87:**4315–4319.
6. **Hayday, A. C., D. J. Diamond, D. Tonigawa, J. S. Heilig, V. Folsom, H. Saito, and S. Tonegawa.** 1985. Unusual organization and diversity of T-cell receptor α-chain genes. Nature (London) **316:**825–832.
7. **Joyner, A. L., W. C. Skarnes, and J. Rossant.** 1989. Production of a mutation in mouse En-2 gene by homologous recombination in embryonic stem cells. Nature (London) **338:**153–156.
8. **Mansour, S. L., K. R. Thomas, and M. R. Capecchi.** 1988. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature (London) **336:**348–352.
9. **Mortensen, R. M., M. Zubiaur, E. J. Neer, and J. G. Seidman.** 1991. Embryonic stem cells lacking a functional inhibitory G-protein subunit (α_{i2}) produced by gene targeting of both alleles. Proc. Natl. Acad. Sci. USA **88:**7036–7040.
10. **Nelson, F. K., W. Frankel, and T. V. Rajan.** 1989. Mitotic recombination is responsible for the loss of heterozygosity in cultured murine cell lines. Mol. Cell. Biol. **9:**1284–1288.
11. **Paludan, K., M. Duch, N. O. Kjeldgaard, and F. S. Pedersen.** 1989. Graduated resistance to G418 leads to differential selection of cultured mammalian cells expressing the neo gene. Gene **85:**421–426.
12. **Potter, T. A., R. A. Zeff, W. Frankel, and T. V. Rajan.** 1987. Mitotic recombination between homologous chromosomes generates H-2 somatic cell variants in vitro. Proc. Natl. Acad. Sci. USA **84:**1634–1637.
13. **Rajan, T. V., L. F. Moffat, and W. N. Frankel.** 1990. Rate and mechanism of generation of beta 2-microglobulin mutants from a heterozygous murine cell line. J. Immunol. **145:**1598–1602.
14. **Schwartzberg, P. L., S. P. Goff, and E. J. Robertson.** 1989. Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. Science **246:**799–803.
15. **te Riele, H., E. R. Maandag, A. Clarke, M. Hooper, and A. Berns.** 1990. Consecutive inactivation of both alleles of the pim-1 proto-oncogene by homologous recombination in embryonic stem cells. Nature (London) **348:**649–651.
16. **Thomas, K. R., and M. R. Capecchi.** 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell **51:**503–512.
17. **Wasmuth, J. J., and L. V. Hall.** 1984. Genetic demonstration of mitotic recombination in cultured Chinese hamster cell hybrids. Cell **36:**697–707.
18. **Zijlstra, M., E. Li, F. Sajjadi, S. Subramani, and R. Jaenisch.** 1989. Germ-line transmission of a disrupted beta 2-microglobulin gene produced by homologous recombination in embryonic stem cells. Nature (London) **342:**435–438.
19. **Zimmer, A., and P. Gruss.** 1989. Production of chimaeric mice containing embryonic stem (ES) cells carrying a homoeobox Hox 1.1 allele mutated by homologous recombination. Nature (London) **338:**150–153.

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