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Effects of Mutation Position on Frequency of Marker Rescue by Homologous Recombination

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Homologous recombination between transferred and chromosomal DNA can be used for mapping mutations by marker rescue, i.e., by identifying which segment of wild-type DNA can recombine with the mutant chromosomal gene and restore normal function. In order to define how much the fragments should overlap each other for reliable mapping, we have measured how the frequency of marker rescue is affected by the position of the chromosomal mutation relative to the ends of the transferred DNA fragments. For this purpose, we used several DNA fragments to effect marker rescue in two mutant hybridomas which bear mutations 673 bp apart in the exons encoding the second and third constant region domains of the immunoglobulin μ heavy chain. The frequency of marker rescue decreased greatly when the mutation was located near one of the ends of the fragments, the results indicating that fragments should be designed to overlap by at least several hundred base pairs. Possible explanations for this "end effect" are considered.

The usual methods for relating molecular structure and function in mammalian systems depend on assaying the activity of transferred DNA. A possible weakness of this approach in studying gene expression has been that the transferred genes were assayed in an abnormal chromosomal context, either integrated at abnormal chromosomal sites or in an unintegrated form. If a regulatory element were to depend on the normal chromosomal context or to lie outside the gene segment that is assayed, the usual gene transfer and expression methods would be inadequate. Homologous recombination between chromosomal and mutant transferred DNA permits us to make predetermined changes in chromosomal genes and thus permits us to test the importance of specific sites for the function of genes in their normal chromosomal location. However, the mammalian genome is vast, so the likelihood of generating mutants with a relevant phenotypic change is impractically low, unless the genetic target of interest is small and well defined. The converse method of selecting mutant organisms according to their phenotypes can also be used to generate informative mutants. In this case, the vastness of the genome makes it impractical to identify the corresponding mutation, unless the mutations can be mapped to a small and well-defined region. A method for mapping mutations is marker rescue, whereby different segments of normal DNA are introduced into the organism to test which segments can recombine with the homologous chromosomal region and restore the normal phenotype. Long fragments are useful for the preliminary localization of the mutation; short fragments are useful for indicating the segment of mutant DNA which must be sequenced. Our previous study of the effect of length measured how the frequency of marker rescue decreased with decreasing fragment length and indicated that overlapping fragments as short as 1 kb yield recombinants sufficient for mapping purposes (6). In that study, the marker was located near the middle of the fragments, so the results did not indicate the extent to which the fragments should overlap. Here we report how the frequency of marker rescue depends on the position of the marker in the fragment. The results indicate how to design suitable overlapping fragments for marker rescue mapping.

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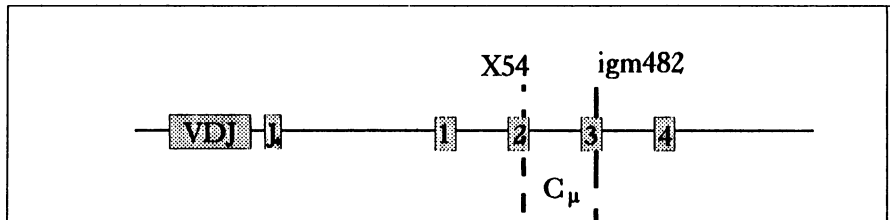
MATERIALS AND METHODS

Tissue culture and cell lines. Techniques and media have been described previously (6). The wild-type hybridoma Sp6 (Sp6/HL subclone), which produces immunoglobulin M(κ) specific for trinitrophenol, and the Sp6-derived mutant igm482 have been described previously (2). Mutant X54 was isolated from Sp6 by a similar suicide selection method and has been found to bear a 2-bp deletion in the exon encoding the second constant region domain (unpublished data).

Gene transfer. The structures of the fragments used for gene transfer are shown in Fig. 2 and 3. The specific fragments of the μ gene were excised by digestion with the indicated restriction enzymes, after which they were isolated by electrophoresis in agarose, electroeluted, and purified by phenol extraction and ethanol precipitation. Hybridoma cells were grown to a density of 1×10^5 to 3×10^5 per ml and 2×10^7 cells were electroporated, as described previously (6). Cell survival was determined by comparing the numbers of viable cells in the electroporated and nonelectroporated cultures after incubating the cells in medium at 37°C for 1 day and ranged from 10 to 70%.

The DNA segments used in this study were derived from the μ gene of the wild-type Sp6 hybridoma and are therefore isogenic with the μ genes of mutants igm482 and X54.

Detection of recombinants. The cells surviving after DNA transfer were expanded so that several plates, each containing approximately 10^7 cells, were assayed for plaque-forming cells (PFC) on trinitrophenol-coupled erythrocytes in the presence of complement 2 and 3 days after electroporation (6). As reported previously (6), there were no significant differences between the PFC measurements made at these two times (data not shown); the results presented here are the averages of the two assays. The frequency of PFC generated in this way has a standard deviation of approximately 30% (5, 6).



	DNA AMOUNT (pmol)	PFC/2x10^7 cells	
		X54	igm482
4.3kb (913bp, 673bp, 2,717bp, XbaI)	30	47	51
	15	42	45
	7.5	19	24
	3.75	4	7
2.4kb (820bp, 934bp, AccI)	30	18	11
	15	23	18
	7.5	11	10
	3.75	3	5
No DNA	0	1	1

FIG. 1. Dependence of marker rescue on amount of DNA.

RESULTS

We have previously described the system used here to measure homologous recombination between transferred and chromosomal immunoglobulin DNA (1, 6). In brief, we make use of mutant cell lines derived from hybridoma Sp6, which has a single copy of the immunoglobulin μ gene and produces immunoglobulin M(κ) specific for the hapten trinitrophenol. The mutants produce abnormal μ chains, and their immunoglobulin M is noncytolytic. This feature provides a convenient assay for distinguishing mutant and wild-type cells. That is, wild-type Sp6 cells make plaques on trinitrophenol-coupled erythrocytes, whereas mutant cells do not. The frequency of marker rescue can thus be measured by the frequency of PFC which result from transferring fragments of the normal μ gene to the mutant hybridomas.

Effect of mutation position on marker rescue. We have used two mutant cell lines in these experiments, X54 and igm482, which bear 2-bp deletions in the exons encoding the second and third constant region domains of the μ heavy chain, respectively (see Materials and Methods). In order to compare different fragments for efficiency of marker rescue, it was necessary to define conditions for which the frequency of marker rescue depended on the amount of DNA. For this purpose, we have used the 4.3-kb *Xba*I and 2.4-kb *Acc*I fragments of the μ gene. As shown in Fig. 1, for both mutants the frequency of marker rescue is approximately proportional to the amount of DNA up to at least 15 pmol, thus defining the range in which fragments can be compared for efficiency of marker rescue. These results also show that the X54 and igm482 cell lines were, as expected, comparable in their propensities for recombination, in that there were no significant differences in the frequencies of marker rescue in these two mutants.

The frequency of homologous recombination between transferred and chromosomal DNA in mammalian cells depends strongly on fragment length (6, 8) (Fig. 1), so in the following experiments we used fragments of comparable length to study the effect of marker position. In a preliminary experiment to test for "end effects," we isolated three fragments of the wild-type μ constant region gene, each approximately 2.8 kb in length and chosen to vary the position of the mutation in igm482 relative to the ends of the fragments (Fig. 2). The results showed that when the mutation was near the end of a fragment, the frequency of recombination was less than when the mutation was near the middle of the fragment.

While these results argued that marker rescue depended on the position of the marker in the transferred DNA fragments, another possible explanation was that the segments of transferred or chromosomal DNA differed in their recombinogenicities. To eliminate this ambiguity, we mea-

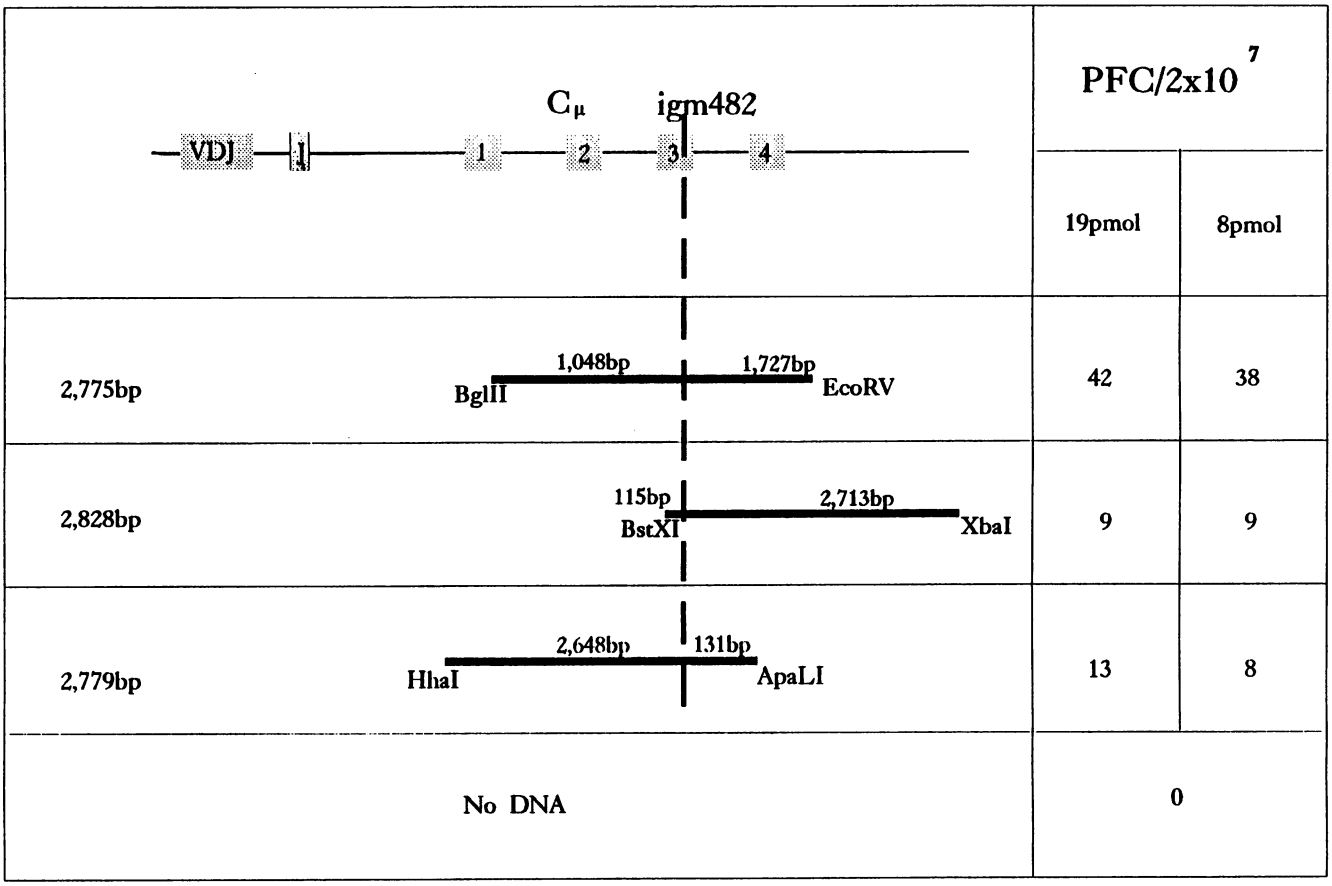


FIG. 2. Evidence for end effects. The top row shows the μ gene of the mutant hybridoma cell line igm482; the dashed line indicates the position of the mutation. The sizes of each fragment and the distances of the mutation from the closer ends are shown. Fragments were prepared and transferred to igm482, and the resulting PFC were measured 2 and 3 days posttransfer, as described in Materials and Methods.

sured marker rescue with the two different mutant cell lines, X54 and igm482, for which the mutations are separated by 673 bp, which allowed us to vary the relationship between marker position and the ends of the fragments.

Figure 3 shows the relationship between the mutations and the fragments which were used to measure the effects of marker position. Seven fragments (A to G) of the wild-type μ constant region gene were isolated and individually transferred to igm482 and X54 by electroporation. Fragments A, B, and C were chosen so that their ends were very close to the X54 mutation, whereas the igm482 mutation corresponded to the middle of these fragments. Fragments D and E were chosen conversely. Fragments F and G were again included because the igm482 and X54 mutations were both near the middle of these fragments. As shown in Fig. 3, the frequencies of recombination for fragments F and G were again nearly the same in both cell lines. By contrast, for the other fragments the frequencies of recombination were lower for the cell line with the mutation near the end of transferred fragment than for the cell line with the mutation near the middle. These results indicate that the frequency of marker rescue is affected by the position of the marker within the fragment.

DISCUSSION

Our results show that the frequency of marker rescue is subject to strong end effects, in that marker rescue is much less frequent when the marker is near an end of the fragment than when the marker is near the middle. The possibility that the cell lines differ in their recombinogenicities is ruled out by the observation that fragments for which both markers were in the middle yielded nearly the same number of PFC with each mutant cell line. Another possibility, that specific sequences of the transferred or target DNA might enhance or depress recombination, is contradicted by the reciprocal nature of our results, namely, that each fragment yielded recombinants at low frequency from one mutant while yielding recombinants at high frequency from the other mutant.

End effects have been investigated in other studies of marker rescue in mammalian cells. Smith and Kalogerakis (7) reported results which showed less recombination when the marker, a 2-kb *neo* gene segment, was near the end of the fragment. In other work, we have measured marker rescue under circumstances for which the crossovers generating PFC could occur in introns; those circumstances permitted us to measure marker rescue with fragments bearing unilateral as well as bilateral homology regions (3). Those experiments revealed that these two types of fragments yielded recombinants at comparable frequencies, provided that the total lengths of homology between the fragments and the target gene were comparable. That is, no end effect was evident in those experiments, presumably because the intron provided a long (6-kb) region in which nonhomologous

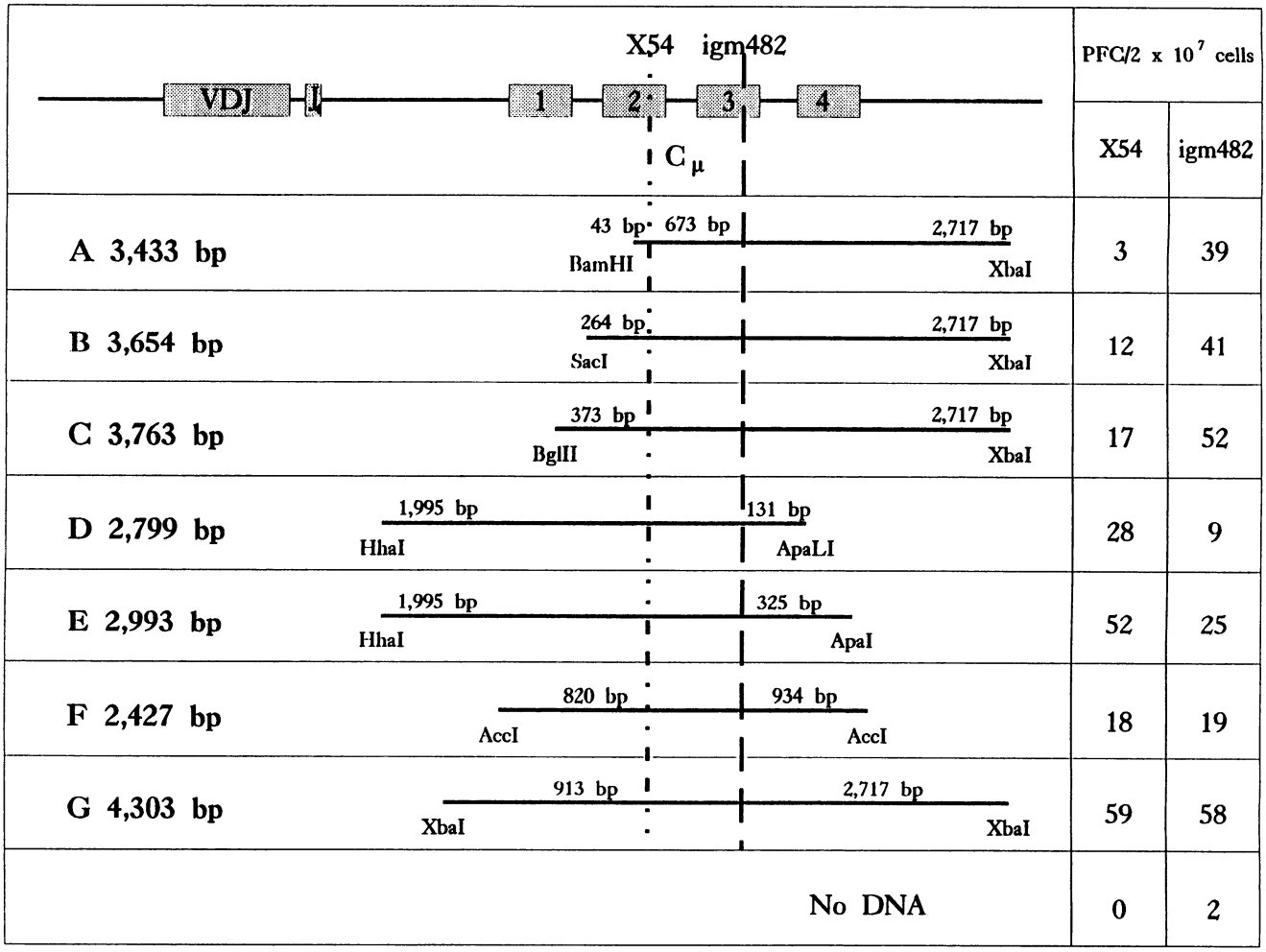


FIG. 3. Effect of mutation position on marker rescue. The top row shows the μ genes of mutant hybridoma cell lines igm482 and X54; the dashed lines indicate the positions of the mutations. The size of each fragment and relevant distances between the mutations and the ends of the fragments are shown. Fragments were prepared and transferred, and the resulting PFC were measured 2 and 3 days posttransfer, as described in Materials and Methods.

recombination could generate PFC, in contrast to the present experiments and those of Smith and Kalogerakis (7), in which the target for nonhomologous crossovers was very short.

We wish to discuss three of the possible explanations for the observed effect of mutation position on frequency of marker rescue. One possibility is based on the observation that mismatches between the transferred and chromosomal μ gene DNAs inhibit the recombination process; if mismatches near the ends of the fragment inhibit more than mismatches near the middle, the mismatches themselves might cause end effects such as those we observed. The other two mechanisms which we discuss suppose that the markers do not interfere with the recombination process. The mechanisms are not mutually exclusive, and other explanations are possible.

The first of these mechanisms assumes (i) that marker rescue depends on the occurrence of two distinct crossovers in the regions to the left and right of the mutation, i.e., in segments L1 and L2 (Fig. 4), and (ii) that the frequency of each crossover is proportional to the segment length. This model thus predicts that the frequency of PFC will be proportional to the product, L1 × L2. Regression analysis of our observations suggests the formulation PFC = 17(L1 × L2), where L1 and L2 are measured in kilobase pairs and PFC is per 2×10^7 cells. As shown in Fig. 4, our results are in reasonable agreement with this hypothesis ($r^2 = 0.82$).

The second model also assumes that recombination depends on two distinct crossovers which occur at a random moment after DNA transfer. In contrast to the above model, we suppose that the recombination occurs only at the ends of the transferred fragment but that the ends of the fragment are degraded exonucleolytically at a fixed rate until recombination occurs. According to this model, the probability that the wild-type sequence will be lost from the left arm of transferred DNA is equal to $e^{-\alpha L1}$, where α is a constant; the probability that the wild-type sequence will not be lost is therefore equal to $1 - e^{-\alpha L1}$. Similarly, the probability that the wild type will not be lost from the right arm of transferred DNA is equal to $1 - e^{-\alpha L2}$. This model thus predicts that the frequency of PFC will be proportional to $(1 - e^{-\alpha L1})(1 - e^{-\alpha L2})$. Analysis of our results suggests the formula PFC = $(1.9 \times 10^7)(1 - e^{-0.00095L1})(1 - e^{-0.00095L2})$. This formula also fits well with our results ($r^2 = 0.84$). Our previous measurements of the relationship between fragment length and

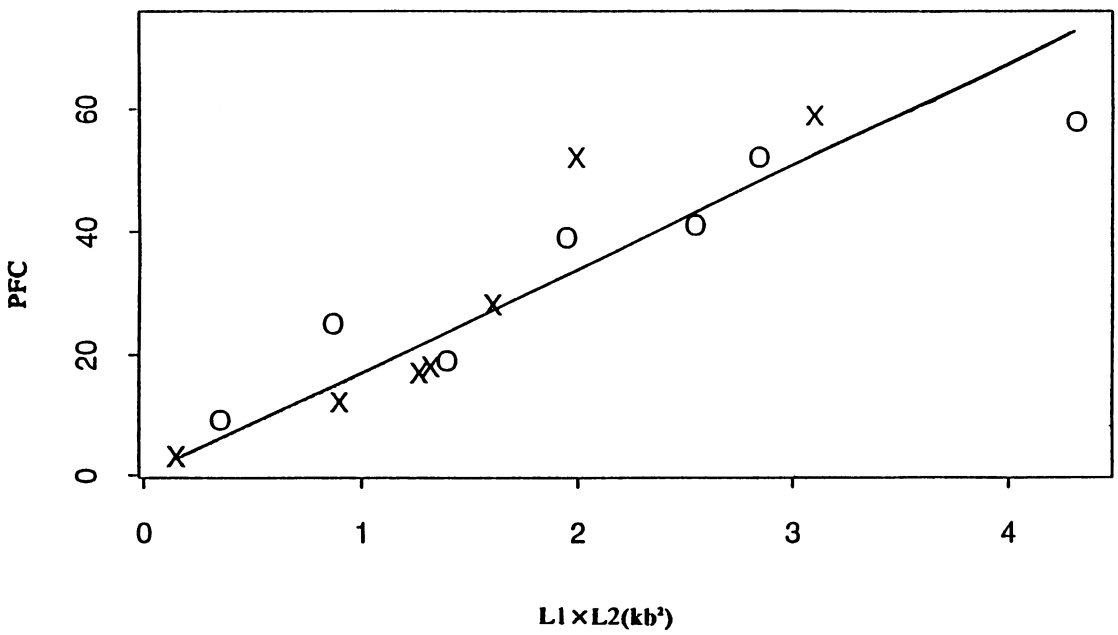


FIG. 4. Comparison of results with model 1. PFC per 2×10^7 cells were measured. O, igm 482; ×, X54.

marker rescue frequency of the immunoglobulin gene are also consistent with both of these formulas (3, 6).

The precedents for exonucleolytic degradation appear to be conflicting. On the one hand, there have been reports which indicate that the ends of transferred DNA can be degraded prior to recombination. Hasty et al. (4) have found evidence that approximately 150 bp are usually degraded during targeted integration events. Exonucleolytic degradation from the cut site might also explain why we recovered fewer wild-type recombinants when the insertion vector was cut closer to the marker (5).

On the other hand, there is evidence against the hypothesis that exonucleolytic degradation reduces the frequency of marker rescue. We have previously shown that the frequency of marker rescue decreases with decreasing fragment length and that this frequency is not increased when fragments are flanked by nonhomologous DNA (6). That is, the decreased recombination observed for shorter fragments was due to their shorter homology region rather than shorter length per se. This result thus argues that exonucleolytic degradation does not interfere significantly with marker rescue, at least not when the marker is as far as 500 bp from the ends of the transferred DNA (6).

To summarize heuristically, our results indicate that marker rescue should be suitable for mapping mutations in the μ gene of these hybridoma cells, under the following conditions. First, there is the implicit requirement that the mutants produce virtually no cytolytic immunoglobulin M, as the marker rescue assay requires detecting very rare PFC. Second, the dependence of the frequency of marker rescue on both the length of homology and the position of the marker can be approximately described by simple formulas. Extrapolating our data with these formulas suggests that in order to detect at least 1 PFC among 2×10^7 recipient cells, the mutation must be more than 20 bp from the ends of 3-kb fragments and more than 60 bp from the ends of 1-kb fragments. Practical consideration demands more than 1 PFC per 2×10^7 recipient cells for reliably scoring marker rescue, so in practice fragments with overlaps of several hundred base pairs should be used.

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REFERENCES

1. **Baker, M. D., N. Pennell, L. Bosnoyan, and M. J. Shulman.** 1988. Homologous recombination can restore normal immunoglobulin production in a mutant hybridoma cell line. Proc. Natl. Acad. Sci. USA **85**:6432–6436.
2. **Bauman, B., M. J. Potash, and G. Kohler.** 1985. Consequences of frameshift mutation at the immunoglobulin heavy chain locus of the mouse. EMBO J. **4**:351–359.
3. **Berinstein, N., N. Pennell, C. A. Ottaway, and M. J. Shulman.** 1992. Gene replacement with one-sided homologous recombination. Mol. Cell. Biol. **12**:360–367.
4. **Hasty, P., J. Rivera-Perez, and A. Bradley.** 1992. The role and fate of DNA ends for homologous recombination in embryonic stem cells. Mol. Cell. Biol. **12**:2464–2474.
5. **Kang, Y., and M. J. Shulman.** 1991. Effects of vector cutting on its recombination with the chromosomal immunoglobulin gene in hybridoma cells. Somatic Cell Mol. Genet. **17**:525–536.
6. **Shulman, M. J., L. Nissen, and C. Collins.** 1990. Homologous recombination in hybridoma cells: dependence on time and fragment length. Mol. Cell. Biol. **10**:4466–4472.
7. **Smith, A. J. H., and B. Kalogerakis.** 1990. Replacement recombination events targeted at immunoglobulin heavy chain DNA sequences in mouse myeloma cells. J. Mol. Biol. **213**:415–435.
8. **Thomas, K., and M. R. Cappechi.** 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell **51**:503–512.