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Stimulation of intrachromosomal homologous recombination in human cells by electroporation with site-specific endonucleases

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ABSTRACT **In somatic mammalian cells, homologous recombination is a rare event. To study the effects of chromosomal breaks on frequency of homologous recombination, site-specific endonucleases were introduced into human cells by electroporation. Cell lines with a partial duplication within the *HPRT* (hypoxanthine phosphoribosyltransferase) gene were created through gene targeting. Homologous, intrachromosomal recombination between the repeated regions of the gene can reconstruct a functioning, wild-type gene. Treatment of these cells with the restriction endonuclease *Xba* I, which has a recognition site within the repeated region of *HPRT* homology, increased the frequency of homologous recombination by more than 10-fold. Recombination frequency was similarly increased by treatment with the rare-cutting yeast endonuclease PI-*Sce* I when a cleavage site was placed within the repeated region of *HPRT*. In contrast, four restriction enzymes that cut at positions either outside of the repeated regions or between them produced no change in recombination frequency. The results suggest that homologous recombination between intrachromosomal repeats can be specifically initiated by a double-strand break occurring within regions of homology, consistent with the predictions of a double-strand-break-repair model.**

Rates of homologous recombination at any given locus in somatic mammalian cells are generally low, on the order of 10^{-8} to 10^{-5} per cell per generation, and seem to vary somewhat from locus to locus within a genome (1). Similarly, frequencies of gene targeting are usually low and have varied greatly from locus to locus (for representative citations, see ref. 2). The basis for the variability and low frequency is not clear.

From studies in bacteria and fungi, the idea has emerged that homologous recombination is often a response to DNA damage in the form of breaks across one or both strands of the DNA duplex. This has been the basis of mechanistic models for recombination proposed by Meselson and Radding (3), Resnick (4), and Szostak *et al.* (5). Many of the central predictions of these models have been borne out by experiments in yeast (*Saccharomyces cerevisiae*). Most notable is that introduction of DNA double-strand breaks within a given chromosomal locus can specifically increase the frequency of homologous recombination at that locus. Natural examples of this in yeast are the initiation of mating-type switch by HO endonuclease and the movements of mobile introns that encode "homing endonucleases" such as I-*Sce* I, I-*Sce* II, and PI-*Sce* I.

In each instance, homologous recombination in the form of gene conversion is triggered by an endonuclease that induces a double-strand break in the recipient locus (6–10). The importance of double-strand breaks for the initiation of homologous recombination in yeast has been extended and generalized by constructing strains in which cleavage sites for the HO or I-*Sce* I endonucleases have been placed within test loci at other chromosomal locations or on extrachromosomal plasmids. Endonuclease expression in such strains sharply elevates the frequency of homologous recombination at the test loci (11–15).

More recently, the I-*Sce* I endonuclease of yeast has been expressed in mammalian cells and shown to stimulate homologous recombination between transfected extrachromosomal DNA substrates (16) or between a transfected substrate and a chromosomal locus (17, 18). In the work reported here, bacterial restriction endonucleases and the PI-*Sce* I endonuclease of yeast have been introduced into immortalized human fibroblasts by electroporation to determine their effects on frequency of homologous recombination between tandemly repeated regions within a chromosomal locus, the *HPRT* (hypoxanthine phosphoribosyltransferase) gene.

MATERIALS AND METHODS

Tissue Culture. All cell lines were derived from the human fibrosarcoma line HT1080 (19) (ATCC no. CCL121). Tissue culture media, antibiotics, and selective drugs were obtained from Sigma. For routine passage and expansion, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 9% fetal calf serum (JRH Biosciences, Lenexa, KS), penicillin (60 μg/ml), streptomycin (100 μg/ml), and selective drugs as described below. In enzyme-electroporation experiments, cells were placed in MCDB302 medium (20), supplemented with serum and antibiotics as above to improve the cloning efficiency of HT1080 cells. Cultures were grown at 37°C, under a humidified atmosphere with 5% CO_2, in plastic tissue culture dishes (Corning).

Gene Targeting. Targeting vectors were made using a 6.9-kb *Hin*dIII fragment of the human *HPRT* gene, which includes exons 2 and 3 and contiguous intron sequences (see Fig. 1). This fragment was inserted at the *Hin*dIII polylinker site of pTZ18R (Pharmacia LKB). A neomycin-resistance cassette from pMC1neopA+ (Stratagene) was inserted between the *Sal* I and *Bam*HI polylinker sites of pTZ18R. Small insertions were then made at an *Xho* I site within *HPRT* exon 3. Vector pE3Hs has a 35-bp insertion with sequence 5′-TCGAGTTATGGGACTACTTCGCGCAACAGTATAAC-3′. Vector pE3Vs has a 66 bp insertion with sequence 5′-TCGATGACGCCATTATCTATGTCGGGTCCGGAGAAAGAGGTAATGAAATGGCAGAAGTCTTGATGG-3′. Targeting vectors were introduced by electroporation using a Bio-Rad Gene Pulser with 0.4-cm gap cuvettes. Cells were trypsinized and resuspended in phosphate buffered saline (137 mM NaCl/8 mM Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4, pH 7.2) to a density of 13.3×10^6 cells/ml, and chilled on ice. One electrical pulse was given at settings of 125 μF and 250 V. Cells were dispersed into chilled DMEM and then plated at 1×10^6 cells

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per 10-cm dish. For selection of targeted cells, the neomycin analog G418 was added at 400 μg/ml 1 day after electroporation; medium was replaced with 6-thioguanine added at 5 μg/ml after 6 days.

Introduction of Endonucleases into Cells by Electroporation. Restriction endonucleases and storage buffer ("diluent buffer A") were obtained from New England Biolabs. PI-*Sce* I was expressed in *Escherichia coli* and purified as described previously (21). The storage buffer for PI-*Sce* I was 10 mM KH_2PO_4, pH 7.6/50 mM KCl/2.5 mM 2-mercaptoethanol/50% glycerol.

Electroporations were done using a Bio-Rad Gene Pulser with 0.4-cm gap cuvettes. Hs-9 and Vs-1 cell lines were expanded under preselection in 6-thioguanine at 10 μg/ml to suppress the accumulation of spontaneous *HPRT*$^+$ recombinants. Preselected cells were trypsinized, washed, and resuspended in phosphate-buffered sucrose (7 mM KH_2PO_4, pH 7.4/1 mM $MgCl_2$/272 mM sucrose) to a density of 8×10^6 cells/ml, and chilled on ice. Chilled cell suspension (150 μl) was mixed thoroughly in an electroporation cuvette with 50 μl of endonuclease in storage buffer or storage buffer alone. One electrical pulse was given at 125 μF and 300 V. The electroporated cells were immediately dispersed into chilled medium. Individual experiments comprised five electroporations from a single population of harvested cells, four with endonuclease present at a range of concentrations and one with enzyme storage buffer only. For selection of *HPRT*$^+$ recombinants, cells were plated initially without drugs at densities of 2.5×10^5, 1×10^6, or 4×10^6 cells per 10-cm dish. After 48 hr, the medium was replaced and hypoxanthine 10^{-4} M, aminopterine 4×10^{-7} M, thymidine 1.6×10^{-5} M (HAT) was added. Medium and HAT were replaced again 4 days later. For determinations of cloning efficiency, cells from each electroporation were plated at densities of 250, 1000, or 4000 cells per 10-cm dish and were not refed. Twelve days after electroporation and plating, both sets of plates were fixed and stained, and cell colonies were counted. Before fixation and staining of selection dishes, one or a few individual HAT-resistant colonies were transferred to separate dishes for expansion. Preparation of genomic DNA, digestion with restriction endonucleases, Southern blotting, and autoradiography were done by standard methods (22, 23).

RESULTS

Construction of an *HPRT* Test Locus in a Human Cell Line. Gene targeting was used to modify the *HPRT* gene in HT1080 cells for use as a test locus. Two targeting vectors, pE3Hs and pE3Vs, were constructed so that their integration at the chromosomal *HPRT* locus would produce a partial duplication within the gene; the repeated regions were separated by plasmid sequence and a neomycin resistance marker (Fig. 1). Each targeting vector also includes a small insertion within *HPRT* exon 3, containing stop codons that break the *HPRT* reading frame. The insertion in vector pE3Vs also contains a cleavage site for the endonuclease PI-*Sce* I. Before transfection, the targeting vectors were linearized by digestion with *Xba* I. HT1080 cells were transfected with pE3Hs or pE3Vs, and after double drug selection for gain of *neo* function and loss of *HPRT* function, multiple targeted clones were isolated. Their structure was confirmed by Southern blotting and PCR analysis (data not shown). Individual clones designated Hs-9 and Vs-1 were chosen for the experiments described below.

The partially duplicated *HPRT* test locus can undergo homologous recombination, as shown in Fig. 1. A reciprocal exchange or "crossover" between the tandem repeats can reverse the targeting to produce a restored, wild-type *HPRT* locus from which the repeat and associated plasmid sequences have been removed. These events can be scored and recovered for analysis using HAT selection for cells that have regained *HPRT* function. They occur spontaneously in both the Hs-9 and the Vs-1 cell lines. We looked for changes in the frequency of crossovers after treatment of Hs-9 or Vs-1 cells with endonucleases that cleave specific sites within the *HPRT* test locus.

Effects on Recombination of Treatment with Restriction Enzymes. We examined the effects on recombination of treatment with five restriction enzymes having sites in the *HPRT* test locus (Fig. 1). We chose one enzyme, *Xba* I, that has a site within the repeated region of the test locus. We also chose two enzymes, *Eco*RV and *Bsp*120I, that cut only outside

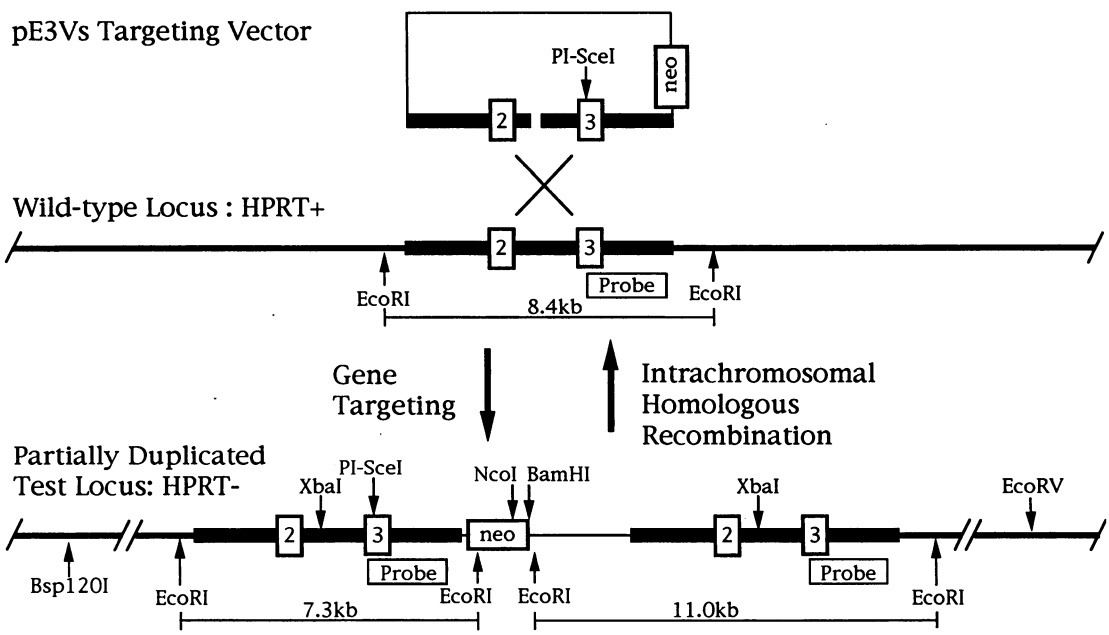

FIG. 1. Construction of an *HPRT* test locus. An *HPRT* test locus was created by targeted recombination between vectors pE3Hs or pE3Vs (pE3Vs is shown) and the wild-type *HPRT* locus of HT1080 cells. Endonuclease cleavage sites within the test locus are indicated. *Nco* I and *Bam*HI have sites within the *neo* gene, 870 bp and 1160 bp away from its left end. For *Eco*RV, the nearest site is 7 kb away from the repeated region in the downstream direction. For *Bsp*120I, the nearest site is 4.5 kb away in the upstream direction.

of the repeated regions and two enzymes, *Bam*HI and *Nco* I, that do not cut within the repeated regions but have sites lying between the repeats. Hs-9 cells were electroporated with 40, 200, 1000, or 5000 units of restriction enzyme or with enzyme storage buffer only. The treated cells were plated for scoring of cloning efficiency and recombination frequency (see *Materials and Methods*).

Viability of the electroporated cell populations, as measured by cloning efficiency, was reduced by any of the five restriction enzymes relative to cells electroporated with enzyme storage buffer only (Fig. 2). Relative cloning efficiency declined in a dose-dependent manner, dropping by 80–95% after treatment with 5000 units of enzyme. The loss in cell viability presumably results either directly or indirectly from chromosomal damage due to endonuclease cleavage (24).

Recombination frequency within the *HPRT* test locus was increased by treatment with *Xba* I (Fig. 3). Frequency increased in a dose-dependent manner to a maximum, after treatment with 5000 units of *Xba* I, of more than 11-fold relative to cells treated with storage buffer only. Of the other four restriction enzymes tested, *Eco*RV, *Bsp*120I, *Bam*HI, and *Nco* I, none had any apparent effect on recombination.

Effects of PI-*Sce* I on Frequency of Recombination. Vs-1 and Hs-9 cells were electroporated with 4, 20, 100, or 500 units of PI-*Sce* I, or with PI-*Sce* I storage buffer only, and plated for measurement of cloning efficiency and for selection of *HPRT*$^+$ recombinants (see *Materials and Methods*). In both cell lines, cloning efficiency trended downward slightly as the amount of PI-*Sce* I used increased, but much less dramatically than with restriction enzymes (Fig. 2). PI-*Sce* I specifically recognizes and cleaves a site that spans about 30 bp (10). Some sequence

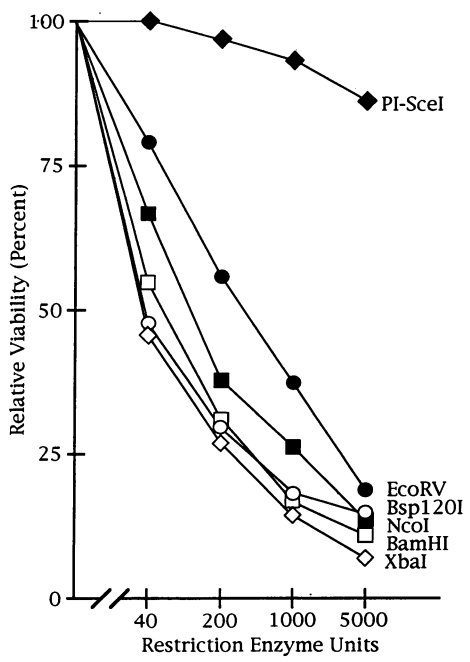

FIG. 2. Effects of restriction enzymes or PI-*Sce* I on viability of Hs-9 and Vs-1 cells. Cell viability was assessed by a cloning efficiency assay. For Hs-9 cells electroporated only with restriction enzyme storage buffer, cloning efficiency was typically ≈20%. For Vs-1 or Hs-9 cells electroporated with PI-*Sce* I storage buffer only, cloning efficiency was typically ≈12%. The relative viability of endonuclease-treated cells was calculated as the ratio of their cloning efficiency to that of cells electroporated with storage buffer only and was expressed as a percentage. Points graphed represent mean relative viabilities for 3 experiments each with *Bsp*120I and *Nco* I, for 5 experiments each with *Bam*HI, *Eco*RV, and *Xba* I (in Hs-9 cells), and for 12 experiments with PI-*Sce* I (4 in Hs-9 cells and 8 in Vs-1 cells). For clarity, error bars are omitted; however, they were comparable to those shown in Figs. 3 and 4.

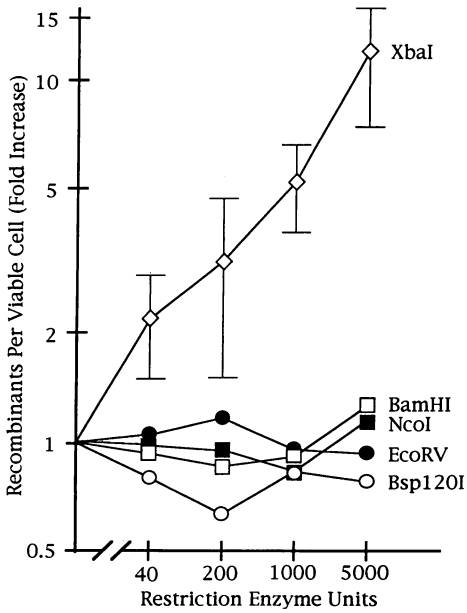

FIG. 3. Relative recombination frequencies in Hs-9 cells electroporated with restriction enzymes. Recombination frequency was calculated as the number of *HPRT*$^+$ colonies scored per million cells plated for selection, divided by the cloning efficiency of the treated population. For Hs-9 cell populations electroporated with no restriction enzyme (with storage buffer only), recombination frequencies averaged 1.8×10^{-4} per viable cell plated. Points graphed represent mean fold increases for three experiments each with *Bsp*120I and *Nco* I and for five experiments each with *Bam*HI, *Eco*RV, and *Xba* I. For clarity, error bars are shown only for the *Xba* I data; errors associated with the other data points are comparable.

variation is tolerated by the enzyme; even so, cleavage sites for PI-*Sce* I are probably rare in the human genome. In Vs-1 cells, which contain a cleavage site within the repeated region of the *HPRT* test locus, recombination was stimulated in a dose-dependent fashion by treatment with PI-*Sce* I. Frequency of recombination increased relative to cells treated with storage buffer only, up to a maximum of nearly 10-fold after treatment with 500 units (Fig. 4). As a control for nonspecific effects, PI-*Sce* I was used in parallel experiments to treat Hs-9 cells, which have no site in the test locus. No effects on recombination frequency were detected (Fig. 4).

DNA Analysis of *HPRT*$^+$ Colonies. Hs-9 or Vs-1 cells that have recovered *HPRT* function through homologous recombination should have a locus structure identical to wild type. DNAs from individual *HPRT*$^+$ colonies were examined after *Eco*RI digestion by Southern blotting, using a probe that distinguishes between the parental duplication and the wild-type locus (Fig. 1). Twenty-eight *HPRT*$^+$ colonies that arose in the absence of enzyme treatment gave the wild-type pattern expected for homologous recombinants, as did 71 of 75 colonies that arose after enzyme treatment (Table 1). Twenty-eight *Xba* I-treated colonies that gave the wild-type pattern were also shown by Southern blotting to retain the *Xba* I site, as expected for gene reconstruction by homologous recombination.

Four of the 75 *HPRT*$^+$ colonies that arose after enzyme treatment gave Southern patterns that did not correspond to that expected for homologous recombinants (Table 1). Reconstruction of the *HPRT* gene in these cells presumably involved a nonhomologous recombination event. The two such colonies that arose after PI-*Sce* I treatment each gave a single, nonwild-type band on Southern analysis (larger in one case, smaller in the other), consistent with gene reconstruction through deletion. The other two colonies gave more complex

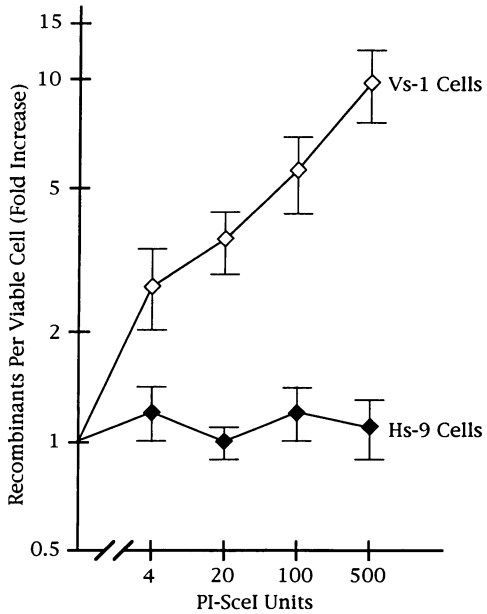

FIG. 4. Recombination frequency in Vs-1 and Hs-9 cells electroporated with PI-*Sce* I. Recombination frequency was calculated as the number of *HPRT*$^+$ colonies scored per million cells plated for selection, divided by the cloning efficiency of the treated population. For Vs-1 cells electroporated without PI-*Sce* I (with storage buffer only), recombination frequencies averaged 0.3×10^{-4} per viable cell plated. For Hs-9 cells electroporated with storage buffer only, recombination frequencies averaged 1.6×10^{-4} per viable cell plated. Points graphed represent mean fold increases for eight experiments in Vs-1 cells and for four experiments in Hs-9 cells. Vs-1 cells have a PI-*Sce* I cleavage site in the *HPRT* test locus; Hs-9 cells do not.

patterns not readily interpretable; they have not been analyzed further.

DISCUSSION

We have shown that intrachromosomal homologous recombination can be stimulated in human cell lines by treatment with site-specific endonucleases. The stimulation appears to be dependent upon the positioning of cleavage sites within homology. Recombination was stimulated by the restriction endonuclease *Xba* I, which has sites lying within repeated regions of homology, but was not stimulated by any of four restriction endonucleases that have sites lying outside the repeated regions or between them. The rare-cutting yeast endonuclease PI-*Sce* I also stimulated recombination when its cleavage site was placed within one repeat, but had no effect when its site was not present.

An alternate explanation for the effect of *Xba* I is that it frequently cuts both repeats in the test locus, and that the outermost ends are then rejoined, with deletion of all sequence between the two cuts, to reconstruct a functional *HPRT* gene. But end joining in mammalian cells is often imprecise (25), and if reconstruction of the *HPRT* gene after *Xba* I cleavage came about mainly by end joining, then at least some of the resulting clones would probably fail to recreate an *Xba* I site at the junction. That all 28 colonies examined retain the *Xba* I site is more consistent with gene reconstruction by homologous recombination. For PI-*Sce* I in Vs-1 cells, reconstruction of an apparently wild-type locus by end joining is not plausible, because the PI-*Sce* I site is installed in only one of the repeats (Fig. 1).

Table 1. Summary of Southern blot analysis of *HPRT*$^+$ colonies

Endonuclease*	Cell line*	No. analyzed	Wild-type pattern†	Aberrant pattern†
None	Hs-9	14	14	0
Xba I	Hs-9	29	28	1
*Bam*HI	Hs-9	24	23	1
None	Vs-1	14	14	0
PI-*Sce* I	Vs-1	22	20	2

*Individual *HPRT*$^+$ colonies were isolated after electroporation of Hs-9 and Vs-1 cells with or without endonucleases.

†Southern patterns of *Eco*RI fragments were classified as matching the wild-type *HPRT* locus (expected for gene reconstruction by homologous recombination) or as aberrant (resulting from other than a simple homologous event).

Results with *Eco*RV, *Bsp*120I, *Nco* I, and *Bam*HI suggest that breaks must occur in the repeated segments to stimulate homologous recombination. All four enzymes reduce the viability of a treated cell population in a dose-dependent way, suggesting that they enter cells and cleave chromosomal DNA. In addition, electroporation of *Eco*RV has previously been shown to cause gross chromosomal rearrangements and mutations at the *APRT* locus in CHO cells (26). The absence of stimulation by these enzymes rules out an indirect or nonspecific effect in which chromosomal breaks trigger a global induction or activation of the enzymatic machinery that carries out recombinational repair.

That *Bam*HI and *Nco* I have no effect on the frequency of *HPRT*$^+$ colonies is somewhat surprising, because cleavage between the repeats might be expected to bring about a reconstruction of the *HPRT* gene through either of two potential pathways. One would be a single-strand annealing pathway, which is one kind of homology-mediated recombination. If cleavage by *Bam*HI or *Nco* I between the *HPRT* repeats were followed with recision of both ends by a strand-specific exonuclease, then eventually single-stranded regions of complementary sequence would be exposed. Annealing of the complementary strands followed by repair would generate a perfectly reconstructed, wild-type *HPRT* locus. Single-strand annealing is the principal pathway for homologous recombination between substrates transfected or injected into vertebrate cells (27–32), and it has also been detected as a pathway of homologous recombination between repeated chromosomal sequences in yeast (33–35). The second potential pathway involves end joining after exonucleolytic recision of DNA from the original cleavage site. An event of this kind could produce an imperfectly reconstructed but nonetheless functional *HPRT* gene. Mammalian cells are known to be highly proficient in end joining (25), and chromosomal deletions have been shown to result from electroporation of mammalian cells with restriction enzymes (26) or from expression of the yeast endonuclease I-*Sce* I (17, 36). If such pathways operate in Hs-9 cells, they are not efficient enough to change the overall frequency of *HPRT*$^+$ colonies after *Bam*HI or *Nco* I treatment. It may be that the particular *Bam*HI and *Nco* I sites between the *HPRT* repeats are not very accessible to the enzymes, so that cleavage at these positions is rare. Or it may be that cleavage is frequent, but end joining usually occurs before enough recision has taken place to allow reconstruction of a functional gene.

Two recent reports, from Rouet *et al.* (17) and Choulika *et al.* (18), have described expression of the yeast endonuclease I-*Sce* I in mouse cell lines to create site-specific chromosomal breaks and have shown that the breaks can stimulate homologous recombination between the chromosome and a transfected targeting vector. In each of these studies, the frequency of recombination was increased by two to three orders of magnitude over controls. In our experiments, treatment with *Xba* I or PI-*Sce* I produced only about a 10-fold increase in homologous recombination frequency. Lukacsovich *et al.* (36) have described the direct electroporation of I-*Sce* I enzyme together with a targeting vector into mouse cells. Although cleavage and repair of the chromosomal target took place, no

homologous recombination between the targeting vector and the chromosomal target was detected. Godwin *et al.* (37) electroporated the restriction enzyme *Pae*R7I into hamster cells containing defective heteroalleles of a reporter gene, but detected only nonhomologous repair of the chromosomal cleavage sites and no homologous recombination between chromosomes. In our experiments, most of the events recovered are apparently homologous; only a minority seem to have involved nonhomologous repair. The differences between these various results may have to do with differences in recombination substrates, the endonucleases chosen, or the methods used to introduce endonucleases into the cell.

Our results are consistent with models of Resnick (4) and of Szostak *et al.* (5), in which the initial step of homologous recombination is proposed to be a double-strand break within one of the homologs that are to recombine. For yeast, it has become clear that initiation in the form of a double-strand break can be a rate-determining step in homologous recombination (6, 7, 10–15). The results reported here, particularly taken together with the results of Rouet *et al.* (17) and of Choulika *et al.* (18), show that initiation by a double-strand break can have the same importance in mammalian cells.

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