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Modification of DNA ends can decrease end joining relative to homologous recombination in mammalian cells

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ABSTRACT **In animal cells, exogenous DNA recombines into random chromosomal sites much more frequently than it recombines into homologous sites. Free DNA ends are "recombinogenic" in both processes. To test the effects of specific ends on analogous extrachromosomal processes, we constructed a linear genome of simian virus 40 with terminal repeated sequences. After transfection into monkey cells, the model substrate can circularize by end joining (analogous to random integration) or by homologous recombination between its terminal repeats (analogous to targeted recombination). Since the two types of recombination are in competition with one another, the ratio of homologous-recombination to end-join products is a sensitive indicator of the differential effects of specific ends. Substrates with blunt ends, complementary sticky ends, or mismatched ends generated the same ratio of homologous-recombination to end-join products. However, addition of dideoxynucleotides to the 3′ hydroxyls of the substrate decreased the frequency of end joining by a factor of 5–6 relative to homologous recombination. Thus, the frequency of end joining can be decreased relative to that of homologous recombination by modification of the ends of the input DNA. These results suggest an approach to altering the ratio of random to targeted integration in mammalian cells.**

In animal cells, the targeted recombination of exogenous DNA at homologous chromosomal locations is masked by a 100- to 1000-fold higher frequency of random integration at nonhomologous positions in the genome (1–7). As a consequence, gene-disruption and gene-replacement experiments of the sort that are commonplace in yeast are impractical in mammalian cells at present (8–10). Thus, random integration interferes with several important objectives: (*i*) introduction of altered genes into their normal chromosomal environment for investigating mechanisms of gene expression, (*ii*) interruption of normal genes in animals for the development of models of human disease, and (*iii*) correction of defective genes as one approach to gene therapy in humans. A means to decrease random integration would be useful for achieving these goals.

Although the mechanisms of targeted and random recombination are undefined, both processes are stimulated by introducing double-strand breaks into the input DNA (1–3, 11). This stimulation suggests that free DNA ends are "recombinogenic" for targeted and random recombination. However, DNA ends probably promote the two processes in different ways. Free DNA ends are thought to stimulate targeted recombination by allowing exposure of single strands, which can then pair with homologous chromosomal sequences (12–15). By contrast, free DNA ends appear to stimulate random integration by permitting direct joining of input DNA ends to chromosomal DNA, perhaps at transient chromosomal breaks. Analysis of randomly integrated DNA molecules suggests that they preferentially integrate through their ends (3, 11) and that the integration event requires very little, if any, nucleotide sequence homology (16–18). In addition, extensive studies with extrachromosomal DNA molecules indicate that mammalian cells, unlike bacteria or fungi, are proficient at joining a wide variety of DNA ends (19–24).

If free ends play different roles, it may be possible to block random integration without interfering with targeted recombination. Such a procedure would be useful for lowering the background of random integration in targeted recombination experiments. To model the role of ends in random and targeted integration, we constructed a modified genome of simian virus 40 (SV40) that would allow us to follow analogous extrachromosomal recombination processes in monkey cells. The model substrate is a linear molecule with terminal repeated sequences, which can circularize by end joining or by homologous recombination between its terminal repeats. Since these two processes are in competition with one another (25), the ratio of homologous-recombination products to end-join products is a sensitive indicator of the differential effects of specific end modifications. In this study we measured the ratio of homologous-recombination products to end-join products as a function of different types of restriction ends and different types of end modification.

MATERIALS AND METHODS

Cells, Virus, and DNA. COS-1 cells (26) were obtained from Randy Kaufman. The SV40 mutant su1903 was derived from wild-type strain Rh911a by replacing a portion of the T-antigen intron with a *Bgl* II linker (24). DNA was labeled *in vivo* with [^{3}H]thymidine and prepared as described (22). Specific activities were measured and used to adjust DNA concentrations for each experiment.

Duplication Substrates. Substrates with terminal duplications were derived from plasmid pXB635 (Fig. 1). In this plasmid the 526-base-pair (bp) *Hin*dIII fragment in the second exon of the T-antigen gene is duplicated (indicated by arrows in Fig. 1), and the *Pst* I fragment encompassing the ends of the T-antigen gene and the *VP1* gene is deleted. The SV40 backbone of the plasmid is attached through its duplicated segments to a synthetic polylinker, whose sequence is indicated in Fig. 1. This polylinker is joined to the polylinker region of the vector, pUC8:2, through mutual *Sma* I sites. Substrates with terminal duplications were cleaved from pXB635 using restriction enzymes with sites in the polylinker. A substrate containing an internal duplication was generated by cleaving plasmid pXB335 (Fig. 1) with *Eco*RI. All substrate molecules were purified from agarose gels before transfection.

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Abbreviation: SV40, simian virus 40.

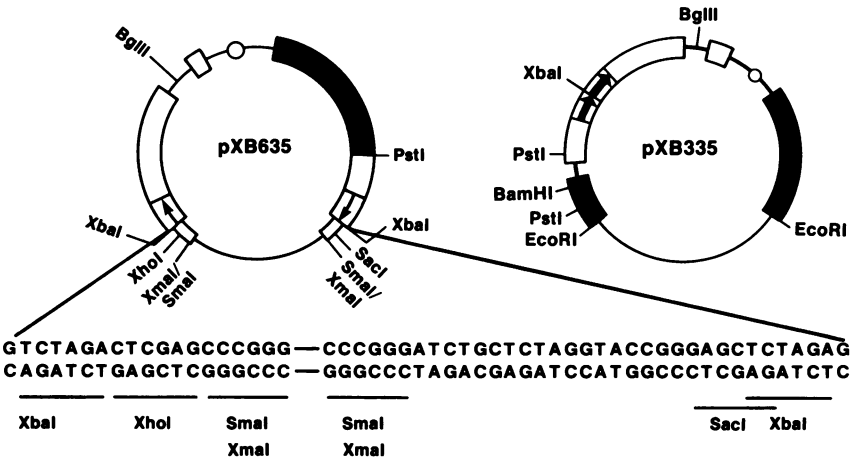

FIG. 1. Structure of the plasmids pXB635 and pXB335. pXB635 was the source of constructs with terminal duplicated segments; pXB335 was the source of constructs with internal, tandem duplicated segments. Arrows indicate the position of the repeated segment. Exons of the T-antigen gene are shown as large open rectangles; capsid genes are represented by filled rectangles. The small circle represents the SV40 origin of replication. The sequence of the polylinker and the positions of relevant restriction sites are indicated below pXB635.

Modification of DNA Ends. Substrates lacking 5′ phosphates were prepared by cleavage with *Xba* I followed by treatment with calf intestinal phosphatase. Substrates with blocked 3′ hydroxyls were prepared by cleavage with *Xba* I followed by incubation with reverse transcriptase in the presence of dideoxycytidine 5′-triphosphate (ddCTP). Ten micrograms of linear DNA was incubated with 4000 units of reverse transcriptase (Moloney murine leukemia virus) at 42°C in the presence of 100 μM ddCTP. DNAs treated with phosphatase or reverse transcriptase were then incubated with T4 DNA ligase to remove molecules with unmodified ends. (In general, <1% of the phosphatase-treated molecules and <10% of the reverse transcriptase-treated molecules could be ligated.) The unligated linear DNA was purified from agarose gels and used in the experiments. The extent of dideoxynucleotide addition in the experimental samples was further assessed by incubating the treated, agarose-purified DNAs with reverse transcriptase in the presence of [α-^{32}P]dCTP. The treated samples incorporated <5% of the radioactivity incorporated into untreated *Xba* I-cleaved control DNA, indicating that <5% of the ends were unblocked.

DNA Transfection and Analysis. DNA transfections used DEAE-dextran as carrier (27). In each experiment 40 ng of substrate DNA was transfected onto COS-1 cell monolayers on 100-mm Petri dishes. Viral DNA was harvested 48 hr after transfection and analyzed by blot hybridization as described (28).

RESULTS

Experimental Design. To measure the effects of different kinds of ends and end modifications on end joining and homologous recombination, we constructed a test substrate with a terminal duplication. We then measured the frequency with which it circularized by end joining or by recombination between its terminal repeats. Homologous recombination generates a circular product with a single copy of the repeat, whereas end joining generates a circular product with two copies of the repeat (Fig. 2). These two products can be readily distinguished by blot hybridization after cleavage with appropriate restriction enzymes (Fig. 2). Several features of the experimental design are relevant.

(*i*) Substrates with different ends have different lengths of nonhomologous sequences at their termini (Fig. 1). The longest segment at any one end is 32 bp. Homologous recombination in mammalian cells is not affected by short, terminal nonhomologies in this size range (13).

(*ii*) The deletion in the C-terminal portion of the T-antigen gene prevents formation of functional T antigen by either means of circularization. However, the supply of T antigen in COS-1 cells renders this region of SV40 nonessential. Since both products depend on the cellular supply of T antigen, their ratio of formation should not be significantly distorted by the replicative amplification necessary for detection.

(*iii*) The defect in the *VP1* gene prevents packaging of the products into viral capsids, thereby confining the products to the initially infected cells.

(*iv*) The probe used to detect the products does not contain

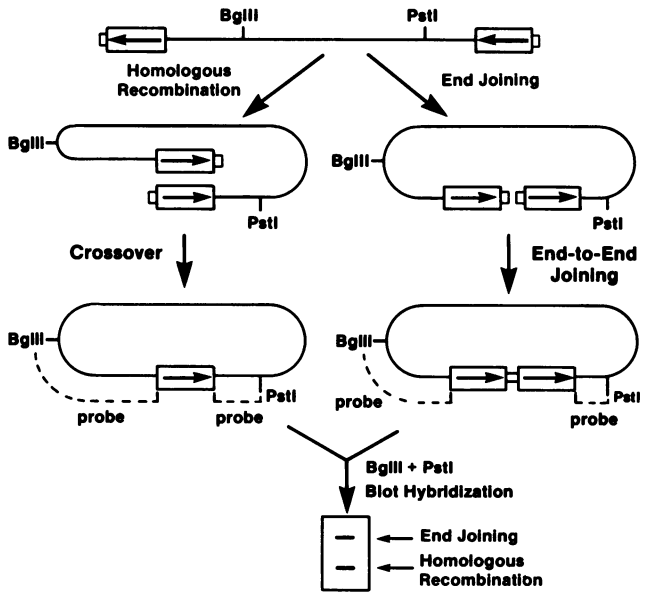

FIG. 2. Schematic diagram of assay to measure the ratio of homologous recombination to end joining. The 526-bp terminal repeats are indicated by the rectangles that contain arrows. The small rectangles at the ends represent variable lengths of nonhomologous sequences, which ranged from 4 to 32 bp. End joining and homologous recombination generate diagnostic restriction fragments, 1.9 and 1.4 kilobases (kb) in length, respectively. The diagnostic fragments were visualized by autoradiography after blot hybridization. The hybridization probe was a nick-translated pUC8:2 plasmid containing the segment indicated by the dashed line. The repeated sequences were deleted from the probe during cloning by *Hin*dIII digestion, which cleaves exactly at the ends of the repeat.

the duplicated segment (Fig. 2). Thus, it should hybridize equally to the two products.

Effects of Sticky, Blunt, and Mismatched Ends on the Ratio of End Joining to Homologous Recombination. Six different substrate molecules were generated by cleaving pXB635 with different restriction enzymes. A substrate with complementary sticky ends was generated by cleavage with *Xba* I; a substrate with blunt ends was generated by cleavage with *Sma* I; and four substrates with a variety of mismatched ends were generated by cleavage with different pairs of restriction enzymes. These molecules were transfected into COS-1 cells and viral DNA was harvested after 48 hr. Viral DNA was restricted with *Bgl* II and *Pst* I, the fragments were separated by electrophoresis on agarose gels, and the fragments diagnostic for the two circularization products were visualized by blot hybridization and autoradiography. Representative gels are displayed in Fig. 3. In all cases the upper band represents the end-join product and the lower band represents the homologous-recombination product (see Fig. 2). To facilitate comparison of band intensities, 1:2 dilutions of the viral DNA were run in adjacent lanes (dilutions are indicated by the numbers above the lanes). Ratios of homologous recombination to end joining were essentially the same for all six substrate molecules (Table 1). Thus, a variety of restriction ends do not affect the relative frequency of end joining and homologous recombination.

Intramolecular Recombination Between Tandem Repeats. Since an end-join product contains a tandem repeat, an apparent homologous circularization product could be generated after end joining by intramolecular recombination between the repeated segments. To evaluate this possibility, we transfected into COS-1 cells a linear molecule that contained two 526-bp segments arranged as an internal tandem repeat. This molecule was derived from pXB335 (Fig. 1) by cleavage with *Eco*RI. The results of this transfection (Fig. 4*D* and Table 1) indicate that about 10% of the molecules with internal tandem repeats were converted into molecules with a single 526-bp segment. This frequency of recombination after end joining is small enough that it does not

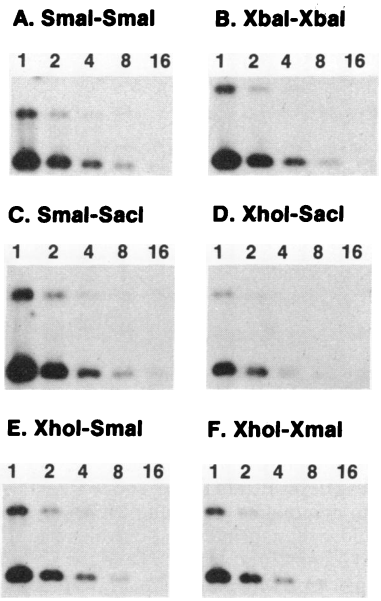

FIG. 3. Effects of sticky, blunt, and mismatched ends on the relative frequencies of end joining and homologous recombination. Viral DNA was harvested 48 hr after transfection, digested with *Bgl* II and *Pst* I, fractionated by electrophoresis on 0.7% agarose gels, transferred to Zetabind membranes, hybridized with nick-translated probe, and visualized by autoradiography. In all cases, the upper band corresponds to the product of end joining and the lower band corresponds to the product of homologous recombination. Bands appear at slightly altered relative positions in the different panels because electrophoresis was carried out for different times. Numbers above each lane indicate dilutions of a sample relative to the first lane.

Table 1. Ratios of homologous recombination relative to end joining

Substrate	Restriction digestion	Treatment	Ends	Ratio*
1	*Sma* I–*Sma* I	None	$-CCC^{OH}$ $^{P}GGG-$ $-GGG_{P}$ $^{OH}CCC-$	4.8 ± 0.6
2	*Xba* I–*Xba* I	None	$-T^{OH}$ $^{P}CTAGA-$ $-AGATC_{P}$ $^{OH}T-$	4.4 ± 0.7
3	*Sma* I–*Sac* I	None	$-CCC^{OH}$ $^{P}C-$ $-GGG_{P}$ $^{OH}TCGAG-$	3.8 ± 0.5
4	*Xho* I–*Sac* I	None	$-C^{OH}$ $^{P}C-$ $-GAGCT_{P}$ $^{OH}TCGAG-$	4.1 ± 0.3
5	*Xho I–Sma* I	None	$-C^{OH}$ $^{P}GGG-$ $-GAGCT_{P}$ $^{OH}CCC-$	4.3 ± 1.1
6	*Xho* I–*Xma* I	None	$-C^{OH}$ $^{P}CCGGG-$ $-GAGCT_{P}$ $^{OH}C-$	4.2 ± 0.4
7	*Xba* I–*Xba* I	Calf intestinal phosphatase	$-T^{OH}$ $^{OH}CTAGA-$ $-AGATC_{OH}$ $^{OH}T-$	5.6 ± 1.1
8	*Xba* I–*Xba* I	Dideoxynucleotide addition	$-TC^{H}$ $^{P}CTAGA-$ $-AGATC_{P}$ $^{H}CT-$	23.8 ± 9.0
9	*Eco*RI–*Eco*RI†	None	$-G^{OH}$ $^{P}AATTC-$ $-CTTAA_{P}$ $^{OH}G-$	0.1

*Ratio of homologous recombination to end joining. The intensities of bands were measured by densitometry. The ratio of band intensities was determined by comparing bands of similar intensity. Ratios were averaged from three to five experiments except for substrate 9, which was measured in only one experiment. The large standard deviation associated with substrate 8 is due to the difficulty in comparing very faint bands (see Fig. 4*C*).

†This substrate, which contains an internal tandem duplication, was prepared by cleaving pXB335 with *Eco*RI (see Fig. 1).

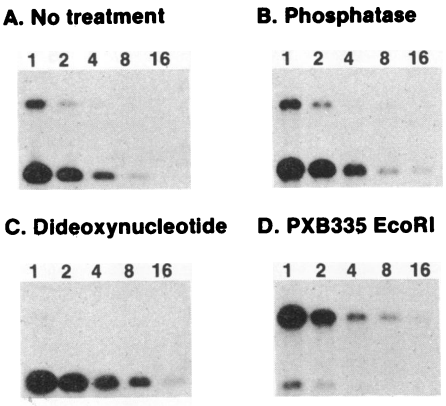

FIG. 4. Effects of modifications of DNA ends on the relative frequencies of end joining and homologous recombination. Analysis was performed as described in the legend to Fig. 3. (*A*) Results from a construct with unmodified *Xba* I ends. (*B*) A construct with *Xba* I ends was treated with calf intestinal phosphatase to remove the 5′ phosphates. (*C*) A construct with *Xba* I ends was treated with reverse transcriptase in the presence of dideoxynucleotides to block the 3′ hydroxyls. (*D*) Results from a construct containing an internal tandem repeat. The construct was derived from pXB335 (Fig. 1) by digestion with *Eco*RI.

significantly affect the experimental ratios of homologous recombination to end joining.

Effect of End Modification on the Ratio of End Joining to Homologous Recombination. The roles of terminal 5′ phosphates and 3′ hydroxyls are not precisely defined in either end joining or homologous recombination in mammalian cells. However, end joining uses the terminal few nucleotides predominantly (24), whereas homologous recombination is insensitive to short terminal nonhomologies (13). Thus, terminal phosphates and hydroxyls might be expected to be more critical for end joining than for homologous recombination. To test this possibility, we modified the ends of an *Xba* I-cleaved substrate, which contains complementary 5′ extensions. The 5′ phosphates were removed from one sample by treatment with calf intestinal phosphatase and the 3′ hydroxyls were blocked on a second sample by addition of dideoxynucleotides. These modified substrates, along with the unmodified control, were transfected into COS-1 cells and the ratios of homologous recombination to end joining were determined by blot hybridization. Results are shown in Fig. 4 and summarized in Table 1. Removing 5′ phosphates had little effect, but blocking 3′ hydroxyls reduced end joining by a factor of 5–6 relative to homologous recombination. Applying both treatments to one substrate was no more effective than addition of dideoxynucleotides alone (data not shown).

DISCUSSION

We have explored the dependence of homologous recombination and end joining on the nature of the ends on exogenous DNA transfected into mammalian cells. To increase the sensitivity of the assay, we placed these two types of recombination in competition with one another by transfecting a linear DNA molecule that could circularize by direct end joining or by homologous recombination between terminal repeated sequences (25). The ratio of the products of homologous recombination and end joining, which can be readily distinguished by restriction digestion, provides a sensitive measure of the differential effects of specific ends.

The ratio of homologous recombination to end joining appears to be independent of the nature of the ends left by digestion with different restriction enzymes (Table 1). Although previous experiments indicated that monkey cells join sticky, blunt, and mismatched ends with the same efficiency as measured by plaque assay (19, 24), it was surprising that joining of all ends occurred at the same rate relative to homologous recombination. Since several mechanisms are used to join different ends (24), it seems unlikely that they would all occur at the same rate. These results suggest that the rate-limiting step in joining DNA ends is not "ligation" *per se*. However, the nature of the rate-limiting step is unclear; it could be apposition of the ends of a molecule or entry of a molecule into a distinct pool destined for end joining (13, 29). Whatever the explanation, the phenomenon probably is not limited to monkey cells since a similar ratio of homologous recombination to end joining (4.1 for a 1.2-kb homology) was determined in human HeLa cells using overlap recombination between adenovirus terminal segments (C. S. H. Young, personal communication).

In previous experiments we demonstrated that terminal nonhomologies in the range from 30 to 325 bp did not noticeably interfere with homologous recombination in monkey cells (13). Substrates 1–6 in Table 1 carried different lengths of terminal nonhomology ranging from 4 to 32 bp (from 8 to 48 bp counting both ends). The constant level of homologous recombination relative to end joining reinforces the conclusion that homology at the exact termini of DNA molecules is not required for homologous recombination in mammalian cells.

The frequency of homologous recombination measured relative to end joining as an internal standard shows a nonlinear dependence on length of homology. In Fig. 5 the ratio of the products of homologous recombination and end joining in monkey cells is shown for three lengths of homology: 131 bp (25), 237 bp (K. Marburger and J.H.W., unpub-

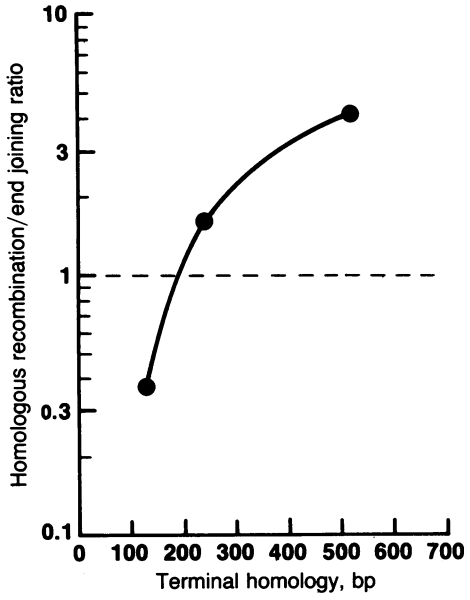

FIG. 5. Ratio of homologous recombination to end joining as a function of the length of terminal homology. Results for substrates with terminal repeats 131 and 237 bp in length were obtained by isolating individual plaques after transfection and characterizing them by restriction digestion (ref. 25; K. Marburger and J.H.W., unpublished). In both cases the ratio of homologous recombination to end joining was determined by comparing the number of plaques that contained virus with monomeric segments to those that contained virus with dimeric segments. The 131-bp segment was located in the T-antigen intron (25). The 237-bp segment, which is defined by the *Bam*HI and *Bcl* I restriction sites in SV40, encompasses the C termini of the *VP1* gene and the T-antigen gene. Genomes with monomers or dimers of the 237-bp fragment have equal viability. (Experiments with the 237-bp terminal repeat used dl884, which carries a 247-bp compensating deletion in the T-antigen intron.)

lished), and 526 bp (Table 1). The ratio of products increases sharply between 131 and 237 bp (4.3-fold increase in recombination per 1.8-fold increase in length of homology) but much less steeply between 237 and 526 bp (2.6-fold increase in recombination per 2.2-fold increase in length of homology). Other studies in monkey cells and human cells have detected a similar break in the dependence of recombination on length of homology in the range of 200–300 bp (30, 31).

Removal of 5′ phosphates had little effect on the rate of end joining relative to homologous recombination. Other studies suggest that 5′ phosphates are efficiently replaced in monkey cells. Removal of 5′ phosphates from the restricted ends of linear SV40 genomes did not decrease the frequency of end joining as measured by plaque assay nor did it alter the frequency with which the restriction site was regenerated upon circularization (D. B. Roth and J.H.W., unpublished). Blocking the 3′ hydroxyls by addition of dideoxynucleotides proved a much more effective method for reducing end joining: it decreased end joining by a factor of 5–6 relative to homologous recombination. How this treatment interferes with end joining is not clear, although the result suggests that dideoxynucleotides are not readily removed. In addition, it is unclear from these measurements whether molecules that did not join end-to-end were circularized by homologous recombination or were simply degraded. Similar experiments using a substrate with a repeat length more favorable to end joining might be able to detect a net increase in the homologous recombination products, if it occurs.

In summary, we have described a model system to measure the extrachromosomal ratio of homologous recombination relative to end joining. This system provides a rapid method for determining whether specific treatments of the input DNA or of the host cells alter the ratio. In this paper we showed that addition of dideoxynucleotides to the 3′ hydroxyls of the input DNA significantly decreased the frequency of end joining relative to homologous recombination. These experiments demonstrate that the ends of the input DNA can be modified in a way that decreases their competence for end joining but does not alter their competence for homologous recombination.

These results are relevant to attempts to target exogenous DNA to homologous chromosomal locations in mammalian cells, which have been hampered by the much more frequent integration of such DNA at random sites (1–7). Random and targeted recombination both depend on free DNA ends. Modification of ends to block end joining (but not homologous recombination) could improve the ratio of targeted recombination to random integration in two ways: (*i*) by preventing the joining of input DNA molecules to one another, end modification would maintain the concentration of free ends at a maximum, which might promote an increase in the absolute frequency of targeted recombination; (*ii*) modification of the ends may block the joining of input DNA to chromosomes, thereby decreasing the frequency of random integration directly. However, since the joining of an input DNA end to chromosomal DNA involves only one blocked end, the effect on random integration is difficult to predict. To model this reaction, we tested a substrate with dideoxynucleotides at only one end. With this substrate, end joining was decreased by a factor of 3 relative to homologous recombination, which is half the effect observed when both ends were modified (data not shown). Overall, our results demonstrate that modification of ends can decrease the frequency of end joining. Dideoxynucleotide addition or other modifications of ends may be useful for decreasing the frequency of random integration.

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