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Genetics

Relative rates of homologous and nonhomologous recombination in transfected DNA

(somatic recombination/end joining/simian virus 40/tandem vs. terminal duplications)

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ABSTRACT **Both homologous and nonhomologous recombination events occur at high efficiency in DNA molecules transfected into mammalian cells. Both types of recombination occur with similar overall efficiencies, as measured by an endpoint assay, but their relative rates are unknown. In this communication, we measure the relative rates of homologous and nonhomologous recombination in DNA transfected into monkey cells. This measurement is made by using a linear simian virus 40 genome that contains a 131-base-pair duplication at its termini. Once inside the cell, this molecule must circularize to initiate lytic infection. Circularization can occur either by direct, nonhomologous end-joining or by homologous recombination within the duplicated region. Although the products of the two recombination pathways are different, they are equally infectious. Since homologous and nonhomologous recombination processes are competing for the same substrate, the relative amounts of the products of each pathway should reflect the relative rates of homologous and nonhomologous recombination. Analysis of individual recombinant genomes from 164 plaques indicates that the rate of circularization by nonhomologous recombination is 2- to 3-fold higher than the rate of homologous recombination. The assay system described here may prove to be useful for testing procedures designed to influence the relative rates of homologous and nonhomologous recombination.**

The various DNA rearrangements known to occur in mammalian cells include homologous events, which depend upon extensive sequence homology, and nonhomologous events, which require little or no sequence homology. Although commonly used, the terms "homologous" and "nonhomologous" are not precisely defined in the literature. Here we use the term "homologous" to describe recombination events that are thought to proceed through a heteroduplex intermediate, and we use "nonhomologous" to describe events that are thought not to utilize such an intermediate. Examples of homologous events include genetic recombination during meiosis (1), sister chromatid exchange (2) and interchromosomal recombination during mitosis (3, 4), and nonallelic gene conversion (5–7). Some examples of events that appear to be nonhomologous include chromosome translocations (8), certain gene amplification events (9), the movements of retroviruses and transposable elements (10), the rearrangements of antibody and T-cell receptor genes (11–14), and the formation of processed pseudogenes (15).

We and others have demonstrated that homologous and nonhomologous events occur readily and at high efficiency among DNA molecules transfected into vertebrate cells (16–36). Our experiments have used the simian virus 40 (SV40) genome as a reporter molecule to probe the recombinational capabilities of cultured monkey cells. Our previous work has shown that linear SV40 genomes with terminal redundancies ranging from 0.5 to 5 kilobase pairs in length circularize by homologous recombination with an efficiency approaching 100% (35). We have also shown that linear genomes with sticky, blunt, or mismatched ends circularize with the same high efficiency (35). These results indicate that homologous and nonhomologous processes are equally efficient when measured by an endpoint assay, but the results do not provide information about relative rates, which could be quite different.

To measure the relative rates of homologous and nonhomologous recombination, we used a linear, terminally redundant SV40 genome that can circularize by either pathway to generate viable progeny virus (Fig. 1). Since the two recombination processes are in competition for the same substrate, the relative amounts of the viral genomes produced by each process should be a measure of their relative rates.

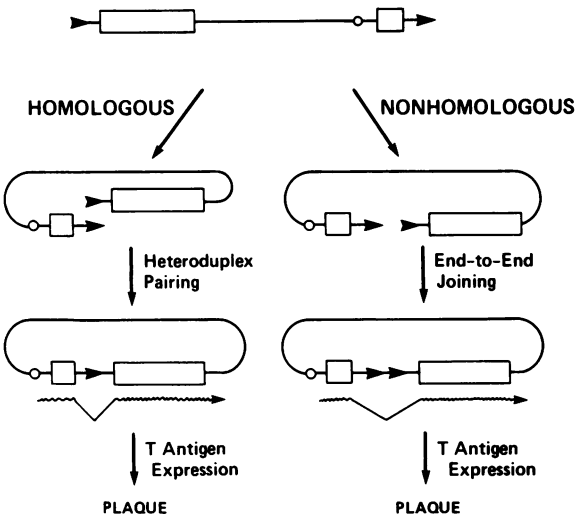

FIG. 1. A kinetic competition assay for measurement of the relative rates of homologous and nonhomologous recombination. At the top of the figure, a linear derivative of SV40 is shown. The 131-base-pair terminal repeats are indicated by arrowheads and the exons encoding tumor (T) antigen are shown as open rectangles. The SV40 origin of replication is designated by a small open circle. After transfection into CV1 cells the linear construct can circularize by homologous recombination, which proceeds by heteroduplex pairing and leads to deletion of one copy of the duplicated region. Circularization can also proceed via direct joining of the ends, leading to retention of both copies of the duplicated region. In both cases, circularization reconstitutes a functional T-antigen gene, and expression of T antigen initiates the infectious cycle and leads to formation of a plaque. Genomes containing either one or two copies of the 131-base-pair region should be equally viable, since the duplication is in the intron of the T-antigen gene, which is removed from the mature messenger RNA as shown. Clonal descendants of the original recombinant molecule can be recovered from an isolated plaque.

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Abbreviations: SV40, simian virus 40; T antigen, tumor antigen.

The substrate used in these experiments contained a terminal redundancy of 131 base pairs that was constructed in the intron of the gene for T antigen, which is required for viral replication and subsequent expression of the late viral genes. This construction accomplished two purposes. First, the T-antigen gene was interrupted, so that T antigen could not be expressed until after circularization, thereby ensuring that the recombination processes responsible for circularization were of cellular origin. Second, the target for recombination was placed in a nonessential region of the genome, so that the products of both homologous and nonhomologous recombination events would be equally viable. To give rise to a plaque, a molecule must circularize by either the homologous or the nonhomologous pathway, and the products of these two pathways can be readily distinguished by restriction mapping. Analysis of viral genomes derived from isolated plaques allowed us to distinguish the products of each pathway, and from this information we determined the relative rates of homologous and nonhomologous recombination. For this substrate, the rate of nonhomologous recombination was 2–3 times the rate of homologous recombination. DNA sequence analysis of some of these products revealed several pathways for end-joining.

MATERIALS AND METHODS

Cells, Viruses, and DNAs. Procedures for the growth of the established monkey kidney cell line CV1 have been described (36). The SV40 mutants used in this work were derived from the Rh911a wild-type strain. The substitution mutant, su1901, has been described (35). In this mutant, a region of the intron of the large T-antigen gene has been replaced by sequences derived from pBR322. SV40 and plasmid DNAs were prepared according to published procedures (35, 37). DNA transfections were carried out as described previously (37), using DEAE-dextran. Plaque assays and viral infections were performed according to published procedures (38). All enzymes were purchased from Boehringer Mannheim and used according to the supplier's recommendations.

Construction of Duplication Substrates. To construct the duplication substrate, we used a mutant of SV40, su1901. Within the substituted intron, unique recognition sites for *Taq* I and *Fnu*DII are separated by 131 base pairs (see Fig. 2). Linear DNA containing a terminal duplication of 131 base pairs was constructed by two different methods as shown in Fig. 2. Both protocols created fragments that overlapped by 131 base pairs at one end; these fragments were ligated together at the other end, generating a terminally duplicated linear genome. Method I gave fragments that were greater than 95% free of full-length species, as judged by agarose gel electrophoresis. The purified fragments used in method II were checked by plaque assay and found to be greater than 99% free of full-length parental DNA. In both methods, the linear construct was purified from other ligation products by preparative agarose gel electrophoresis. This purified material was used for transfection.

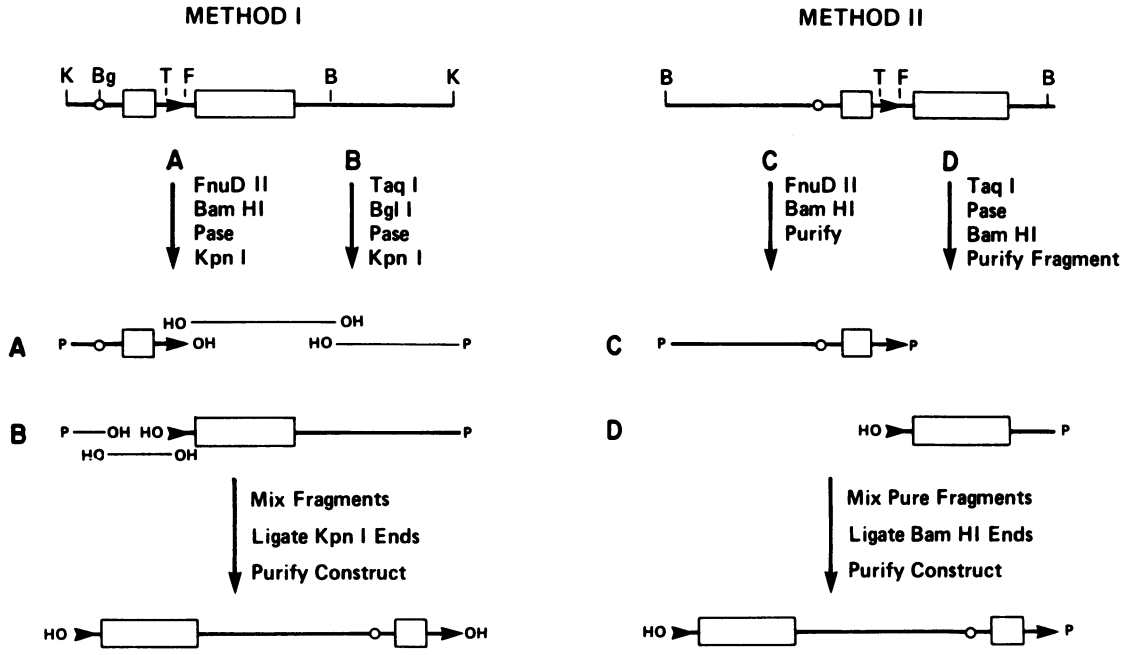

FIG. 2. Construction of linear SV40 DNA containing a 131-base-pair terminal duplication. The substrate for the kinetic competition assay was prepared according to two different methods. Both methods involved isolation of two overlapping fragments of DNA, which were then ligated to form the desired construct. Method I removed the "unwanted" fragments by cleaving them with restriction enzymes and treating with phosphatase, thus preventing their religation. In method I, circular viral DNA (shown here as a linear species for purposes of illustration) was divided into two aliquots. One aliquot was cleaved with *Fnu*DII to generate one terminus of the construct. This DNA was digested with *Bam*HI, which cleaves the larger fragment, and then treated with phosphatase to prevent religation of the *Bam*HI fragments. The phosphatase was removed and the mixture was digested with *Kpn* I. This treatment leaves a mixture of three fragments, two of which contain one phosphorylated end (*A*). The desired fragment is shown on the left. The second aliquot of DNA was digested with *Taq* I to create the other terminus of the construct and then digested with *Bgl* I to cleave the unwanted fragment. The mixture was treated with phosphatase, and then the phosphatase was removed and the fragments were digested with *Kpn* I. The resulting mixture of three fragments is shown in *B*; the desired fragment in this case is shown on the right. Mixtures *A* and *B* were mixed together and ligated at high DNA concentration, and the desired construct was purified from the other ligation products by preparative agarose gel electrophoresis. The only ligation product that contains the entire SV40 genome is the desired construct; none of the other products was infectious.

In method II, the desired fragments were purified by agarose gel electrophoresis prior to ligation. One aliquot of SV40 DNA was cleaved with *Fnu*DII and *Bam*HI, and the fragment shown in *C* was purified. Another aliquot was cleaved with *Taq* I and treated with phosphatase to prevent ligation through the *Taq* I ends. The phosphatase was removed and the DNA was digested with *Bam*HI and the fragment shown in *D* was purified. Fragments *C* and *D* were mixed and ligated, and the desired construct was purified from the ligation mixture by preparative agarose gel electrophoresis.

To arrange the same 131-base-pair region as a tandem repeat, one aliquot of su1901 was linearized by digestion with *Taq* I, and the resulting cohesive ends were filled in by using the Klenow fragment of DNA polymerase I. A second aliquot of su1901 was cleaved with *Fnu*DII, which leaves blunt ends. The two linear blunt-ended fragments were mixed and ligated at high concentration. The resulting mixture of oligomers was cleaved with *Bam*HI, which cleaves SV40 DNA at a unique site. The mixture of digestion products containing the desired construct was then inserted directly into the *Bam*HI site of pBR322, and the molecule of interest was isolated by screening the plasmid library. Prior to transfection, the pBR322–SV40 chimera was cleaved to completion with *Bam*HI to liberate a linear SV40 genome containing an internal duplication of 131 base pairs. The resulting mixture was used for transfection.

Miniwell Preparation of DNA. Confluent monolayers of CV1 cells in 96-well microtiter plates were infected with picked plaque suspensions, and viral DNA was labeled *in vivo* by addition of [^{32}P]orthophosphate as described previously (36).

RESULTS

Relative Rates of Homologous and Nonhomologous Recombination. To assay the relative rates of homologous recombination and nonhomologous end-joining, we construted a linear DNA containing a 131-base-pair terminal repeat (see Fig. 2 for construction schemes). This molecule can be circularized by either homologous or nonhomologous recombination, thus generating products that contain either one or two copies, respectively, of the repeated sequence (see Fig. 1). DNA constructs were introduced into CV1 monkey kidney cells by transfection, and a large number of the resulting plaques were isolated for analysis of viral DNA. The infectivity of this construct (expressed as plaques per nanogram of input DNA) was similar to that of linear, wild-type SV40 DNA (35). This observation is consistent with our expectation that the overall efficiency of circularization would be high.

To determine the structure of the genomes of the recombinant viruses, radiolabeled viral DNA was prepared from individual plaques and analyzed by restriction mapping. An autoradiogram of a representative gel is shown in Fig. 3. The figure shows examples of viral DNAs containing two copies of the 131-base-pair region (marked "dimer") as well as DNAs containing a single copy of the region (marked "monomer"). The lanes marked "deletion" contain viral genomes with major changes, which are due to deletions (unpublished data).

Table 1 shows the classification of the 164 picked plaques analyzed as described above. Most (95%) contained either one or two copies of the 131-base-pair repeat sequence; the rest (5%) had suffered deletions. The deleted genomes presumably arose from nonhomologous end joining after breakage of input molecules, as observed previously (35). These molecules (and presumably a larger number of nonviable genomes) were excluded from the analysis of relative rates of homologous and nonhomologous recombination, because breakage eliminates their ability to compete for homologous recombination processes. The overall ratio of progeny with dimer segments to those with monomer segments is 114/42 = 2.7. This ratio suggests that the rate of nonhomologous end-joining is 2.7-fold higher than the rate of homologous recombination.

However, this conclusion rests upon the assumption that the dimer or monomer segments were generated by the circularization event itself. We have considered two possible alternative sources for the monomer segment: contamination with the DNA used as starting material for construction, and homologous recombination subsequent to end-joining. To address the possibility of contamination, we used two different construction methods (see Fig. 2 for details) and tested the degree of contamination for each method (less than 5% for method I and less than 1% for method II) as described in *Materials and Methods*. Since the measured amount of contamination was small, and since both methods gave almost identical results, we conclude that contamination is not a significant source of progeny genomes containing monomer segments.

A second potential source of monomer segments is the conversion of a dimer segment to a monomer segment by homologous recombination after circularization. To assess the frequency of recombination after circularization, we transfected a construct containing the repeated segment as an internal duplication (see *Materials and Methods*). Labeled DNA preparations derived from 43 of the resulting plaques

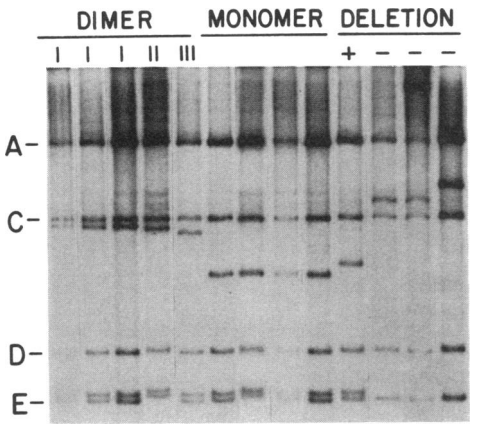

FIG. 3. Restriction enzyme analysis of recombinant genomes. The products of individual recombination events were analyzed by digestion with the restriction enzymes *Hin*dIII and *Taq* I. ^{32}P-labeled viral DNA was prepared from plaque suspensions by the miniwell method. Labeled DNA was digested and electrophoresed through 5% polyacrylamide gels, which were dried and autoradiographed. *Hin*dIII cuts SV40 DNA into five fragments, denoted by the letters A–E. The *Hin*dIII B fragment is absent because it is split by *Taq* I digestion into two subfragments: the smaller of these is 430 base pairs in length and migrates slightly above the E fragment. The larger *Hin*dIII/*Taq* I subfragment contains either a monomer or a dimer of the 131-base-pair region. The presence of a band migrating at the 730-base-pair position is diagnostic for a genome containing one copy of the 131-base-pair region; a band migrating at 860 base pairs indicates the presence of a dimer of the 131-base-pair sequence. These assignments were confirmed by digesting each sample with *Hin*dIII plus *Fnu*DII (not shown). The designations I, II, and III above the lanes marked "dimer" denote electrophoretic variants of the 860-base-pair band (see text). The symbols + and − above the lanes marked "deletion" refer to the presence or absence of the *Taq* I site (in many of the deletions, this site was deleted). All samples in the figure were run on the same gel, but the order of the lanes in the photograph was rearranged for illustration.

Table 1. Analysis of recombinant genomes

Genome type	Event	Plaques: Method I*	Plaques: Method II†	Sum
Monomer segment	Homologous	24	18	42 (25%)
Dimer segment	End-join	55	59	114 (70%)
Other	Break-join	8	0	8 (5%)

*Linear DNA prepared according to method I (Fig. 2) was transfected into CV1 cells (0.04–0.08 ng of DNA per plate) and assayed at 37°C. Plaques were picked and analyzed as in Fig. 3 and classified as shown.

†Linear DNA was prepared according to method II (Fig. 2) and transfected into CV1 cells as in method I.

were subjected to restriction enzyme analysis. As shown in Table 2, none of the genomes contained a monomer segment, indicating that homologous recombination between the internally repeated segments occurred at a very low frequency. As a further test of the stability of the internal duplication, we infected cells with virus containing the duplication. DNA obtained from 70 of the resulting plaques was analyzed by restriction mapping; again, no homologous recombination events were detected (see Table 2). These results indicate that an internally duplicated region of 131 base pairs undergoes homologous recombination at a frequency of less than 1%. Therefore, homologous recombination after circularization is not a significant source of progeny genomes containing monomer segments.

Since neither contamination nor postcircularization recombination events contribute significantly to the relative amounts of the monomer- and dimer-containing products, we conclude that these relative amounts reflect the relative rates of the two processes.

Characterization of Nonhomologous Recombination Products. The ends of the construct used in these experiments are mismatched; the end generated by *Fnu*DII digestion is blunt, whereas the *Taq* I end has a two-nucleotide 5′ extension. Mismatched ends may require modification of one or both ends prior to joining, and such modification might lead to differences in the length of the dimer segment-containing restriction fragment. Careful examination of autoradiograms such as the one shown in Fig. 3 revealed heterogeneity in the electrophoretic mobility of this restriction fragment. We have defined three mobility classes, designated I, II, and III in the figure. Nucleotide sequence analysis of eight representatives from class I, which accounted for about 80% of all molecules that circularized by end-joining, indicates that the 5′ extension of the *Taq* I end was removed prior to joining. In class II, which comprises approximately 13% of end-joined species, the recessed 3′ end generated by *Taq* I digestion was filled in. The modified terminus was then joined to the blunt *Fnu*DII end, fortuitously regenerating the *Fnu*DII restriction site. All members of class II were found to contain a *Fnu*DII site at the end-join junction. The remaining 7% of end-join species have been grouped together into class III, which includes all the recombinants whose junction fragments have an electrophoretic mobility different from class I or class II. The nucleotide sequences we have obtained suggest that the modifications responsible for the variations in electrophoretic mobility observed in class III involved removal of less than 25 base pairs from the ends, a finding consistent with our earlier estimates (35). These data will be presented in detail elsewhere.

Table 2. Stability of tandem repeat

Test	Genome type	Plaques
DNA transfection*	Dimer segment	43
	Monomer segment	0
	Other	0
Viral infection†	Dimer segment	70
	Monomer segment	0
	Other	0

*Viral DNA containing an internal tandem duplication was inserted through its unique *Bam*HI site into the *Bam*HI site of pBR322. This DNA was grown and purified from bacterial cells, and the viral portion was separated from plasmid sequences by digestion with *Bam*HI. This mixture was transfected into CV1 cells (0.04–0.08 ng of SV40 DNA per plate) and assayed at 37°C. The resulting plaques were picked and analyzed as described for Fig. 3.

†A suspension of infectious virus particles was obtained from a picked plaque known to contain a tandem duplication. This suspension was plaque assayed on CV1 cells. The resulting plaques were picked and analyzed as described for Fig. 3.

DISCUSSION

We have used a derivative of the SV40 genome containing a 131-base-pair terminal duplication as a substrate in experiments designed to measure the relative rates of homologous and nonhomologous recombination. As shown in Fig. 1, circularization of the linear DNA construct by homologous recombination results in a product that contains a single copy of the 131-base-pair region. In contrast, circularization by end joining leads to the formation of a product that retains both copies of the 131-base-pair sequence. Since both processes compete for the same DNA substrate, a comparison of the number of progeny genomes containing dimer segments with those containing monomers should accurately reflect the relative rates of nonhomologous and homologous recombination. Analysis of 164 recombinant viral genomes demonstrated that, for this substrate, the rate of nonhomologous end-joining was 2.7 times that of homologous recombination.

We have also measured homologous recombination by using a substrate containing the 131-base-pair region arranged as a tandem, internal duplication; in this case the recombination frequency was less than 1%. These data indicate that homologous recombination within terminal repeats is much more frequent than recombination within tandem repeats of the same length. Thus, the efficiency of the recombination process may be affected by the position of homologous regions relative to DNA ends. Other evidence in support of this conclusion is the low infectivity (0.3% of that of wild-type circular DNA) of circular SV40 genomes containing two 237-base-pair repeats separated by 4.3 kilobase pairs of pBR322 DNA (21). For this construct to generate a wild-type genome, the plasmid DNA must be removed by homologous recombination within the repeated region. The low recombination frequency of this construct, compared with the relatively high frequency of homologous recombination found with the genome containing a terminal duplication, suggests that the position of homologous regions at DNA ends stimulates homologous recombination.

Double-strand breaks stimulate homologous and nonhomologous recombination in yeast (reviewed in ref. 39), and in mammalian cells (25, 28, 30, 31; unpublished data). The mechanism by which double-strand breaks stimulate homologous recombination is not known, although several models have proposed that cellular enzymes, such as exonucleases or helicases, act at DNA ends to expose single-stranded regions, which then initiate homologous pairing (refs. 28 and 39; unpublished data). The stimulatory effect of breaks on nonhomologous recombination may be due to creation of free ends that are substrates for an efficient, indiscriminant end-joining reaction. The recombinogenic nature of free ends suggests that both homologous and nonhomologous processes may function in the cell to repair potentially lethal chromosome breaks (40).

The relatively high rate of nonhomologous recombination may provide a serious obstacle to homology-dependent "targeted integration" of input sequences into the genome. As discussed above, the most favorable substrate for homologous recombination is a molecule that is linearized at or near the region of homology. Indeed, it is known that the introduction of appropriate linear DNA species into mammalian cells stimulates the frequency of homologous integration into the chromosome (23, 31). Since free DNA ends also stimulate nonhomologous recombination, linear substrates can be rapidly converted to circular or multimeric forms by intra- or intermolecular end-joining events. Thus, the end-joining pathway may lower the likelihood of homologous integration by effectively competing for recombinogenic free ends; conditions that decrease the relative rate of end-joining may stimulate homologous integration.

The kinetic competition assay we have described may be a useful model system with which to study the effects of various parameters on the relative rates of the two processes. It can be used to investigate the effects of varying the amount of homology, altering the nature of the DNA ends, and treatments of the host cells. Procedures that increase the relative rate of homologous recombination may ultimately be used to maximize the efficiency of targeted integration into the genome.

Note Added in Proof. Rubnitz and Subramani (41) have recently suggested that a minimum of approximately 200 base pairs of homology is required for efficient homologous recombination in monkey cells. In contrast, we have observed efficient homologous recombination between 131-base-pair terminal repeats (Table 1). This paradox may be explained by differences in the arrangement of the homologous regions in these two experiments: Rubnitz and Subramani assayed for recombination between internal repeats, whereas we assayed for recombination between terminal repeats. When we arranged the 131-base-pair repeats as an internal tandem, the frequency of homologous recombination fell to less than 1% (Table 2), a value in agreement with the measurements made by Rubnitz and Subramani. These results indicate that recombination can occur efficiently between short homologous sequences, if they are situated at or near the ends of a DNA duplex.

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