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Intermolecular Recombination Assay for Mammalian Cells That Produces Recombinants Carrying Both Homologous and Nonhomologous Junctions

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We present an intermolecular recombination assay for mammalian cells that does not involve the reconstitution of a selectable marker. It is based on the generation of a shuttle vector by recombination between a bacterial and a mammalian vector. The recombinants can thus be amplified in mammalian cells, isolated by plasmid rescue in an *Escherichia coli* RecA⁻ host, and identified by in situ hybridization, by using mammalian vector sequences as probes. Since both parental molecules can share defined lengths of homology, this assay permits a direct comparison between homologous and nonhomologous intermolecular recombination. Our results indicate that the dominant intermolecular recombination mechanism is a nonhomologous one. The relative frequency of homologous to nonhomologous recombination was influenced by the length of shared homology between parental molecules and the replicative state of the parental molecules, but not by the introduction of double-strand breaks per se. Finally, almost all of the recombinants with a homologous junction did not have the reciprocal homologous junction but instead had a nonhomologous one. We propose a model to account for the generation of these recombinants.

During the last few years, a number of assays have been developed to study recombination in mammalian cells. In these assays, exogenous DNA is introduced into cultured cells by various methods, such as infection, calcium-phosphate precipitation, DEAE-dextran transfection, microinjection, and protoplast fusion. In most cases, the recombination products are identified by the reconstitution of a selectable marker, as a result of either intra- or intermolecular homologous recombination (2, 3, 5, 8, 11, 12, 17, 20–24, 27, 28, 32–36, 38, 40, 45, 47, 49, 50). Some assays have also looked at nonhomologous recombination (1, 13, 19, 30, 52), and in a few instances, intramolecular recombination assays have been devised that permit comparison of homologous and nonhomologous recombination (6, 31, 37, 39).

The main conclusions from these assays are that cultured cells will readily recombine exogenous DNA by homologous (6, 11, 17, 20, 27, 33–35) and nonhomologous recombination (10, 12) and that when compared in intramolecular assays, both recombination mechanisms have similar frequencies (6, 31, 37, 39). The frequency of homologous recombination depends on the length of homology (3, 18, 21, 32, 34, 39), and it can be significantly increased by introducing a double-strand break in or near the selected homologous sequences (5, 6, 11, 20, 21, 33, 49). This has prompted several authors to propose models for homologous recombination involving a double-strand break (21, 44, 49). Double-strand breaks have also been reported to increase illegitimate recombination (6, 12), which is thought to involve ligation of free ends (12, 48).

In this paper we present a novel recombination assay. This assay is based on the generation of a shuttle vector by intermolecular recombination between a bacterial and a mammalian vector. Parental molecules are cotransfected in mammalian cells, and recombinants are extracted after a 48-h incubation by Hirt fractionation and isolated by plasmid rescue in an *Escherichia coli* RecA⁻ host. Recombinants are identified by in situ hybridization of bacterial colonies by using mammalian vector sequences as probes. Since recombinants produced by both homologous and nonhomologous recombination mechanisms are rescued and since the two parental molecules can share various lengths of homology, this assay permits a direct comparison between homologous and nonhomologous recombination. It is the first intermolecular recombination assay that does so.

Using this assay, we found that the majority of intermolecular recombinants were the result of nonhomologous recombination, even when the parental molecules shared 2.3 kilobase pairs (kbp) of homology. The relative frequency of homologous to nonhomologous recombination increased with the length of homology between parental molecules. Introduction of double-strand breaks in the plasmid vector, as well as at identical positions in both vectors, did not increase the relative frequency of homologous to nonhomologous recombination. However, the relative frequency was increased significantly when double-strand breaks were introduced in the mammalian vector or in both vectors at asymmetrical positions. Finally, the generation of a homologous junction was coupled in almost all cases with the production of a nonhomologous junction.

MATERIALS AND METHODS

Preparation of RmI and construction of plasmid vectors. RmI is composed of a complete polyomavirus genome with an insertion of mouse cellular DNA (Fig. 1). It is an excision product from C12a1, a mouse cell line transformed by polyomavirus mutant *ts*P155 (4, 42, 43). RmI is isolated by Hirt extraction and purified as previously described (4).

Plasmid pLSN6 (Fig. 1) was kindly provided by P. Bourgaux. It contains a 191-base-pair (bp) *Bcl*1 fragment of

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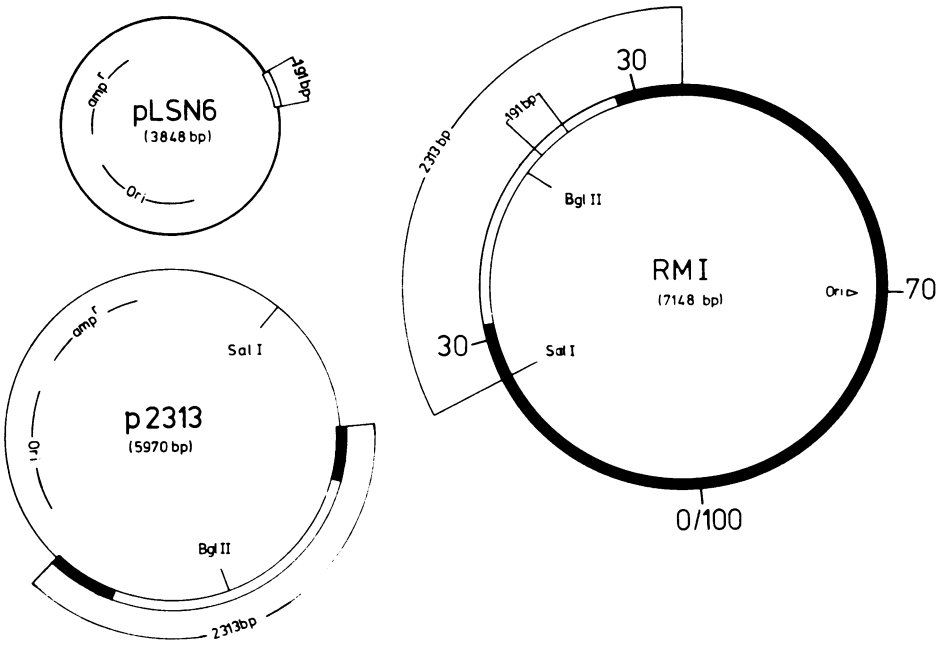

FIG. 1. Parental molecules used in our intermolecular recombination assay. RmI is composed of a complete polyomavirus genome (■) with an insertion (□) in its late region of a segment of 1,628 bp of mouse cellular DNA (4, 42). Ori stands for the origin of replication of polyoma. The numbers refer to standard map units of the polyoma genome. pLSN6 and p2313 are pAT153 plasmids (solid line) with insertions of RmI of 191 and 2,313 bp, respectively. Amp^r is the gene coding for ampicillin resistance, and Ori is the region of the plasmid responsible for autonomous replication.

RmI cloned into the unique *Bam*HI site of pAT153. Plasmid p2313 (Fig. 1) was constructed by inserting a 2,313-bp *Hin*cII fragment of RmI into the unique *Bal*1 site of pAT153.

DNA transfection and bacterial transformation. Mouse 3T6 cells were seeded at 10^6 cells per 90-mm petri dish, 24 h before transfection. Each petri dish was cotransfected with 0.5 μg each of RmI and pLSN6 or p2313 by the DEAE-dextran transfection procedure of McCutchan and Pagano (26), as modified by Sussman and Milman (41). After 48 h, the cells were extracted by the method of Hirt (16). The Hirt supernatants were then phenol extracted, ethanol precipitated, and suspended in 10 mM Tris hydrochloride (pH 7.5)–1.0 mM EDTA. These purified Hirt preparations were used to transform DH1, an *E. coli* RecA$^-$ strain, to ampicillin resistance, as described by Hanahan (15).

Identification of recombinants. All bacterial colonies obtained after transformation were transferred onto nitrocellulose or nylon filters by the method of Grunstein and Hogness (14). Colonies containing recombinant molecules were identified by in situ hybridization with ^{32}P-labeled nick-translated polyomavirus probes. In recombination assays between pLSN6 and RmI, the complete polyoma genome was used as a probe, whereas in recombination assays between p2313 and RmI, only the polyoma sequences not present in p2313 were used as a probe. The nick translation technique was that of Rigby et al. (29), and the hybridization procedures were by van der Ploeg and Flavell (46) with minor modifications described elsewhere (7).

Mapping of recombinants. Colonies with positive hybridization signals were purified, and plasmid DNA was extracted by a small-scale extraction procedure with alkali (25). Restriction endonuclease digestion was performed as recommended by the manufacturers (Amersham Corp., Boehringer Mannheim Biochemicals, New England BioLabs Inc., and Pharmacia Inc.). Digestion products were separated by electrophoresis through horizontal agarose gels and visualized by ethidium bromide staining.

RESULTS

Intermolecular recombination. In our first assay, we cotransfected circular molecules of RmI and pLSN6 which share 191 bp of homology (Fig. 1). Five independent cotransfections were performed, and the resulting Hirt preparations were used in ten different transformation assays. Of the 15,569 transformants generated (Table 1), 44 hybridized with a polyomavirus probe. Thus, 0.3% of the transformants were recombinant molecules. This number was relatively constant with the different Hirt preparations, varying between 0.2 and 0.8%. We did a detailed restriction enzyme mapping of all the recombinant molecules (data not shown). Since our assay permits replication of the recombinants once generated in the mammalian cells, we isolated multiple copies of a number of the recombinants. The 44 recombinants could be divided into 28 unique restriction patterns. None of the 28 recombinants contained an RmI-pLSN6 junction resulting from homologous recombination between their shared 191 bp. In other words, all the RmI-pLSN6 junctions mapped in the 28 distinct recombinants were the result of nonhomologous recombination. Thus, the ratio of homologous to nonhomologous recombination events was less than 0.036. The fact that the recombinant copy number was variable was a strong indication that recombination had occurred in the mouse cells. Nevertheless, to ensure that recombination was not taking place in the bacteria, we transformed the *E. coli* RecA$^-$ host with circular molecules of RmI and pLSN6 (Table 1). We screened 8,450 colonies. None hybridized with the polyoma probe.

In our next assay, we cotransfected mouse cells with circular molecules of RmI and p2313 (Fig. 1) which share

TABLE 1. Intermolecular recombination assay

Parental molecules cotransfected[a]	No. of transformants[b]	% Recombinants[c]	No. of recombinants analyzed		Ratio[d]
			Nonhomologous	Homologous	
RmIcc × pLSN6cc	15,569	0.3	28	0	<0.036
Control	8,450	<0.01			
RmIcc × p2313cc	1,162	5.2	50	7	0.14
Control	14,400	<0.007			
RmIcc × p2313/*Bgl*II	98	63	42	6	0.14
Control	96	<1.0			
RmIcc × p2313/*Sal*I	34	85	24	2	0.08
Control	ND	ND			
RmI/*Bgl*II × p2313cc	458	10	14	8	0.57
Control	7,006	<0.01			
RmI/*Sal*I × p2313/*Bgl*II	63	94	25	17	0.68
Control	ND	ND			
RmI/*Bgl*II × p2313/*Bgl*II	10	100	8	0	<0.13
Control	74	<1.4			

[a] cc indicates that the transfected molecules were in a closed circular form. RmI/*Bgl*II and RmI/*Sal*I are RmI cut at its unique *Bgl*II or *Sal*I sites, and p2313/*Bgl*II and p2313/*Sal*I are p2313 cut at its unique *Bgl*II or *Sal*I sites, respectively. The controls were the same molecules used to transform *E. coli* DH1 directly.
[b] Number of bacterial colonies screened. ND, Not done.
[c] Percentage of transformants that hybridized with the polyoma probe (see Materials and Methods).
[d] Ratio of homologous to nonhomologous recombinants.

2,313 bp of homology (Fig. 1). This is 12 times more homology than there was between RmI and pLSN6. Three independent transfections were performed and used in 14 separate transformation assays. In situ hybridization with a polyomavirus probe consisting of the RmI sequences not present in p2313, was conducted on 1,162 transformants (Table 1). A total of 61 positive signals was obtained, representing a frequency of recombinants per transformants of 5.2%. This frequency varied between 2.2 and 6.8% with the different Hirt preparations. This is 17 times more than the frequency of recombinants per transformants between circular RmI and pLSN6 (Table 1). We do not know the reason for this difference between pLSN6 and p2313. The increase in the frequency of homologous recombinants was not sufficient to explain these results (see below). Indeed we have no indication that this is a true increase in recombination frequency. We believe that it may reflect the various rates at which pLSN6 and p2313 persist in mammalian cells. In agreement with this interpretation, we obtained on average 18 times fewer transformants per transformation assay with RmIcc × p2313cc than with RmIcc × pLSN6cc. The 61 recombinants could be classified in 57 distinct restriction patterns. Of these, 50 contained only nonhomologous junctions between RmI and p2313. The remaining seven contained one of the two homologous RmI-p2313 junctions expected from a single reciprocal homologous crossing-over between RmI and p2313, but the second RmI-p2313 junction had in every case sustained a deletion. We have drawn the recombinant which would result from a single reciprocal homologous crossing-over between RmI and p2313 (Fig. 2A). Also depicted are the deletions observed in the recombinants with one homologous junction. One of the recombinants is not illustrated because its nonhomologous junction had a complex structure that involved not only a deletion but also the insertion of additional RmI sequences.

If we consider that the above recombinants are homologous recombinants as will be argued below, then the ratio of homologous to nonhomologous recombinants in crosses between circular molecules of RmI and p2313 is 0.14 (Table 1). This is at least four times more than the ratio between RmI and pLSN6 and indicates that the length of homology influences the ratio of homologous to nonhomologous recombination. Nevertheless, despite a homology of over 2 kbp, the main mode of recombination remained a nonhomologous one.

Double-strand break. It has been shown that a double-strand break in the homologous sequences increases the incidence of homologous recombination between 10 and 300 times (see Introduction). How would a double-strand break affect the ratio of homologous to nonhomologous recombination? We did the following assays to answer this question.

First, we cotransfected circular RmI with p2313 linearized at its unique *Bgl*II site, which is in the homologous sequences (Fig. 1). Of 98 transformants screened, 62 were recombinants (Table 1). This very high incidence of recombinants per transformants was not due to an increase in recombination frequency. It was due to the fact that by linearizing the plasmid vector, we strongly inhibited its capacity to transform *E. coli*. This was reflected by a strong diminution in the number of transformants obtained. The 62 recombinants belonged to 48 distinct restriction patterns. Of these 48 recombinants, 42 did not have a homologous RmI-p2313 junction. The remaining six had one homologous RmI-p2313 junction with the reciprocal junction having suffered deletions of various lengths (Fig. 2B). In this assay, the ratio of homologous to nonhomologous recombinants was 0.14. This was the same ratio as with circular molecules of RmI and p2313 (Table 1). Thus, the introduction of a double-strand break in the homologous sequences did not increase the ratio of homologous to nonhomologous recombination. This was confirmed by our next assay, in which circular RmI was cotransfected with p2313 linearized at its unique *Sal*I site, which is outside the homologous region (Fig. 1). Of 34 transformants analyzed, 29 were recombi-

nants. These represented 26 distinct restriction patterns. Of those 26 recombinants, 24 had only nonhomologous RmI-p2313 junctions. The remaining two had one homologous RmI-p2313 junction with the reciprocal junction having sustained deletions (Fig. 2C). Thus, the ratio of homologous to nonhomologous recombinants was 0.08.

In a third assay, we cotransfected circular p2313 with RmI cut at its unique *BglII* site (Fig. 1). Of 458 transformants screened, 48 were recombinants, which represents a frequency of 10% (Table 1). The 48 recombinants could be divided into 22 distinct restriction patterns. Of these 22 recombinants, 14 contained only nonhomologous RmI-p2313 junctions. Of the remaining eight recombinants, one was the result of a single homologous crossing-over and thus had the two RmI-p2313 homologous junctions (Fig. 2D, recombinant D176). The other seven had again only one of the homologous RmI-p2313 junctions, with the reciprocal homologous junction having suffered deletions of various lengths. In this assay, the ratio of homologous to nonhomologous recombinants was 0.57. This represents a fourfold increase over the ratio between circular molecules of RmI and p2313 or between circular RmI and linearized p2313. Thus, this effect is not due to the double-strand break per se but probably to the fact that linearization of RmI prevents it from replicating before recombination (see Discussion). It should be noted that the majority of recombinants were still the result of nonhomologous recombination.

In the following assay, RmI and p2313 were cut at asymmetrical sites: RmI at its unique *SalI* site (Fig. 1) and p2313 at its unique *BglII* site (Table 1). This resulted in linear molecules that shared 1,278 bp of homology at one end. Of 63 transformants analyzed, 94% were recombinants. These represented 42 distinct restriction patterns, of which 25 had only nonhomologous RmI-p2313 junctions. The remaining 17 recombinants had one homologous RmI-p2313 junction. They all had the same homologous junction with the reciprocal junction having sustained various deletions, including at least the overlapping homologous sequences between the *BglII* and *SalI* sites. Five homologous recombinants representative of the group are illustrated in Fig. 2E. The ratio of homologous to nonhomologous recombinants was 0.68. This ratio is similar to the one obtained with RmI/*BglII* × p2313cc. Thus, linearization of both parental molecules did not further enhance the relative frequency of homologous to nonhomologous recombination. The fact that all the homologous recombinants contained the same homologous junction suggested that overlapping ends were involved in the generation of homologous junctions. This was confirmed by our last assay, in which both RmI and p2313 were cut at their common *BglII* site (Table 1), thus generating no overlapping ends. All 10 transformants analyzed were recombinants. These represented eight distinct restriction patterns. These eight recombinants had only nonhomologous RmI-p2313 junctions.

Intramolecular recombination. One factor that could have influenced the ratio of homologous to nonhomologous recombinants is that the homologous recombinants, once generated, were subjected to a high incidence of intramolecular homologous recombination. This would have regenerated the initial parental molecules. To test this possibility, we repeated our assay by transfecting only D176, the recombinant that was the result of a single reciprocal crossing-over between RmI and p2313 (Fig. 2B). Forty-eight hours after transfection, we did a Hirt extraction and transformed *E. coli* RecA$^-$. Random bacterial colonies were picked, and their plasmids were analyzed in detail. Of 54 plasmids, 37 were identical to D176, 5 had been involved in intermolecular recombination with another molecule of D176, 11 had been involved in intramolecular nonhomologous recombination, and only 1 plasmid was the original p2313 parental molecule. Thus, intramolecular homologous recombination events did not regenerate the original parental molecules at high frequency. Furthermore, since intramolecular nonhomologous recombination had affected only about 20% of the molecules analyzed, it is not likely to be the reason why, in the intermolecular assays, 39 of the 40 homologous recombinants analyzed (Fig. 2) had sustained a deletion in one of the two expected homologous junctions. For a control, we directly transformed *E. coli* RecA$^-$ with D176. Of 36 separate plasmids analyzed, all were identical to D176.

It should be noted that in the five intermolecular recombinants between D176 molecules, three were homologous recombinants, and two were nonhomologous. Thus, even when there was total homology, almost half of the recombination events were nonhomologous ones.

DISCUSSION

In this paper we present a novel recombination assay. One of its distinct features is that recombination between the parental molecules produces a shuttle vector. Recombinants can thus be amplified in mammalian cells and then isolated by plasmid rescue. Another feature is that recombinant molecules are identified by hybridization in situ by using the nonplasmid parental molecule as a probe. Since homologous sequences can be introduced in the vectors, recombinants produced by both homologous and nonhomologous mechanisms can be rescued in the same assay. We are in fact analyzing a subset of all the recombination events, as specific sequences must remain intact in recombinant molecules to be rescued, namely, the bacterial origin of replication and the ampicillin resistance gene. Furthermore, the presence of the polyomavirus origin of replication possibly confers a selective advantage. However, both homologous and nonhomologous recombinants were subjected to deletions and were probably as likely to lose the abovementioned sequences. Thus, although the absolute frequencies of homologous and nonhomologous recombinations were biased by such a selection, there is no indication that the relative frequencies of homologous to nonhomologous recombinations were.

Intramolecular recombination assays have been devised that permit a comparison of homologous and nonhomologous recombination (6, 31, 37, 39), but ours is the first intermolecular recombination assay to do so. In the intramolecular assays, homologous and nonhomologous recombination occurred at similar frequencies. In our intermolecular assay, nonhomologous recombination was at least seven times more frequent than homologous recombination, even after linearization of the plasmid vector.

Using this assay, we found that the ratio of homologous to nonhomologous recombination is dependent on the length of homology shared between the parental molecules. This is as expected from published results from other laboratories that have shown that the frequency of homologous recombination in itself is proportional to the length of homology (3, 18, 21, 32, 34, 39).

Double-strand breaks have been shown to increase the incidence of homologous recombination by 10- to 300-fold (5, 6, 11, 20, 21, 33, 49). In our assay, double-strand breaks per se had little effect on the ratio of homologous to

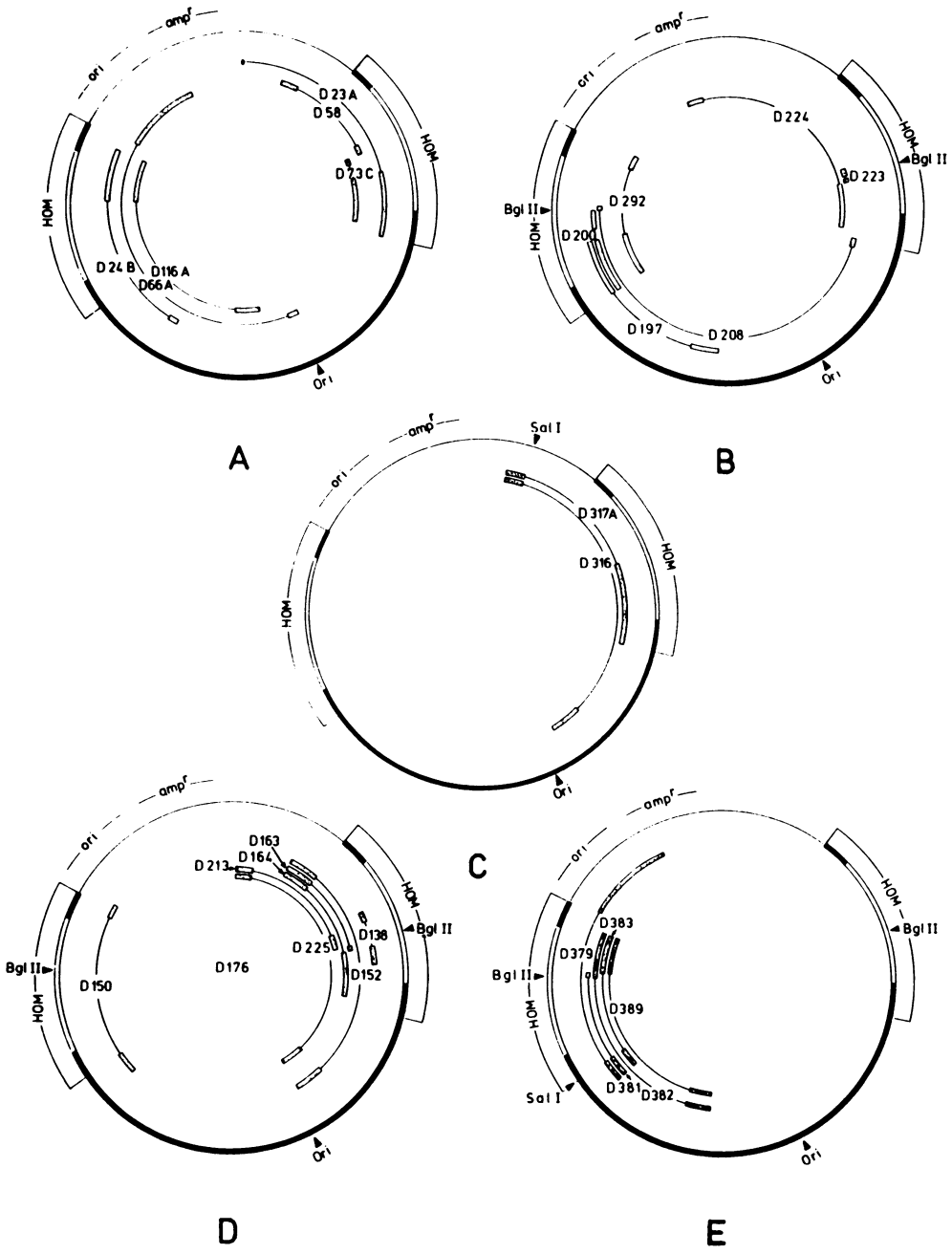

FIG. 2. Maps of homologous recombinants. The structure depicted in all panels is the one resulting from a single reciprocal cross-over between the homologous sequences of RmI and p2313 (Fig. 1). This would generate two homologous junctions that are indicated as HOM. Also indicated inside each circular molecule are the deletions observed in the homologous recombinants identified by specific numbers. The line represents the sequence known to be deleted, while the dotted bars (▨) represent the regions of uncertainty defining the limits of the deletions. Recombinant D176 had no deletion. Each panel represents the homologous recombinants isolated from the cotransfection of RmIcc × p2313cc (A), RmIcc + p2313/*Bgl*II (B), RmIcc × p2313/*Sal*I (C), RmI/*Bgl*II × p2313cc (D), and RmI/*Sal*I × p2313/*Bgl*II (E).

nonhomologous recombination. There was a fourfold increase in the ratio when RmI was cut, but we did not see any increase when p2313 was cut or when both molecules were cut at the same site. We believe that the effect observed when RmI is cut is probably due to another parameter, such as the replicative state of the molecule at the time of recombination. We and others have shown that replication has an influence on the nature and frequency of recombination (9, 51). Why did double-strand breaks not increase the ratio of homologous to nonhomologous recombination? This could be due to the fact that double-strand breaks increase both homologous and nonhomologous recombination to the same extent, as has been observed in intramolecular recombination (6). However, we did not see a significant increase in the total frequency of recombinants when double-strand cuts were introduced (Table 1, RmIcc × p2313cc and RmI/*Bgl*II × p2313cc). Another possible explanation is be that double-strand breaks were very readily produced in our

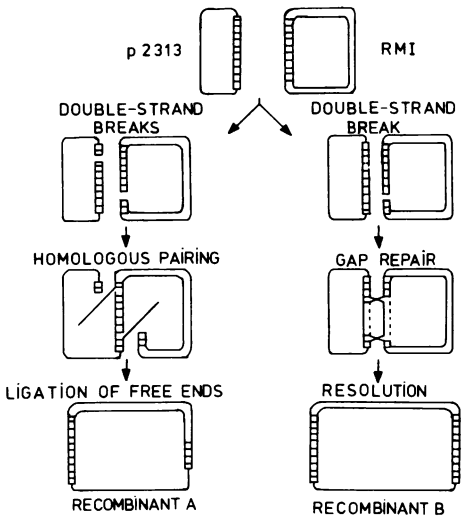

FIG. 3. Model for the generation of homologous recombinants in our intermolecular assay. The region of homology is represented by a dashed bar (▭). On the left-hand side, the pathway involves a double-strand break in both parental molecules. This permits the formation of a homologous junction between the free ends within overlapping homology, possibly by a mechanism as proposed by Lin et al. (21) or Wake et al. (49). The remaining free ends are then ligated to form recombinant A. On the right-hand side, only one parental molecule has a double-strand break. This leads to formation of homologous junctions by a gap repair mechanism as proposed by Szostak et al. (44), which results in recombinant B.

assay, such that they were not a limiting factor to the recombination process. In any event, we note that even with double-strand breaks in the homologous sequences, nonhomologous recombination was still the most frequent mechanism of intermolecular recombination.

In this study, we analyzed by restriction enzyme mapping 28 nonhomologous recombinants between pLSN6 and RmI and 180 between p2313 and RmI. When considering the length of RmI sequences present in the recombinants, we found that there was a wide variation ranging from a few hundred base pairs to almost the entire 7,100-bp molecule, with a uniform distribution of the various lengths (data not shown). This suggests that if nonhomologous recombination is the result of end-to-end ligation as has been suggested (12, 48), then the vast majority of parental molecules are subjected to degradation or breakage before ligation. Another consideration is that the homologous sequences of both parental molecules can be readily maintained in a nonhomologous recombinant. Furthermore, these homologous sequences can be found very close to one another (data not shown). This suggests either that homologous and nonhomologous recombinations are mutually exclusive pathways or that in mammalian cells there is not the same strong homology-seeking mechanism between DNA molecules as has been previously observed in bacteria and yeast cells.

Among a total of 40 recombinants carrying a homologous junction that were generated in the various assays (Fig. 2), only one was the product expected from a single reciprocal crossing-over event between the homologous sequences of the two parental molecules. All others had a deletion in the reciprocal homologous junction. How could such molecules be generated? One possible explanation is that the initial event was intermolecular nonhomologous recombination followed by intramolecular homologous recombination. This hypothesis seems unlikely, however, since about half of the intermolecular nonhomologous recombination events would have had the homologous sequences aligned in opposite orientations. The subsequent intramolecular homologous recombination would have left the homologous sequences in an inverted orientation. This was not observed.

A second possibility is that the first event was intramolecular nonhomologous recombination in one of the parental molecules, followed by intermolecular homologous recombination. This explanation also appears unlikely for several reasons. First, there is no obvious reason for this to happen in the majority of intermolecular homologous recombinants. Second, a number of the deletions seen at the homologous junctions extend from one parental molecule to the other (Fig. 2; D66A, D152, D225, D316, D379). Finally, why would the deletion necessarily involve the homologous sequences in all cases?

A third hypothesis is that the first event was intermolecular homologous recombination leading to a recombinant like D176, followed by intramolecular nonhomologous recombination causing the deletion. We also have to reject this hypothesis because 39 of the 40 recombinants (98%) had a deletion. When we used D176 to evaluate the frequency of intramolecular nonhomologous recombination (see Results), we found it to be about 20%. Thus, this hypothesis could account for only a small proportion of the recombinants. In fact, we believe recombinants D197, D200, and D213 (Fig. 2) could have been generated this way, because these three recombinants have regenerated the restriction site used to introduce a double-strand break in the parental molecule.

This leaves one last alternative. Homologous and nonhomologous recombinations both occurred at the time of intermolecular recombination. We illustrate in Fig. 3 what we believe to be the chain of events. We suppose that in most cases recombination was between two parental molecules with double-strand breaks. The fact that cotransfection with circular molecules gives homologous recombinants with the same overall structure, as with one or both parental molecules being linear, suggests that double-strand breaks are part of the recombination mechanism. Wake et al. (48) have evaluated that each 5 to 15 kbp of transfected DNA is subjected to a double-strand break. If these double-strand breaks are in or near the homologous sequences, this could lead to pairing between homologous sequences sharing an overlap, resulting in a junction of the type proposed by Lin and colleagues (21) and Wake and colleagues (49). To generate such junctions, overlapping homologous ends are necessary for homologous pairing. For RmI/*Bgl*II × p2313/*Bgl*II (Table 1), there was no overlap of the ends, and in fact no homologous recombination was observed, although the number of recombinants screened was small. However, when double-strand breaks were introduced to create overlapping homologous ends (RmI/*Sal*I × p2313/*Bgl*II), all the resulting homologous recombinants had the structure predicted by the model (Fig. 2E; and recombinant A in Fig. 3). Another important aspect of the generation of such homologous junctions is that the degradation upon pairing precludes the formation of the reciprocal homologous junction. For the first time, our assay permits us to look at both junctions on the same recombinant molecule and thus to verify this prediction. We found it to be the case for 39 out of the 40 homologous recombinants (Fig. 2). Since the deletions we observed varied in length, we presume that before ligation, the remaining free ends are either degraded or submitted to another double-strand break.

Since in a few cases homologous recombination led to a reciprocal cross-over (D176 and possibly D213, D197, and D200 [see above]), we conclude that homologous recombinants can be generated by an alternative pathway in which only one parental molecule has undergone a double-strand break (recombinant B in Fig. 3). This leads to a junction of the type proposed by Szostak et al. (44) in their double-strand gap repair model. Thus, both pathways can occur in the same cells.

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