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The Minimum Amount of Homology Required for Homologous Recombination in Mammalian Cells

JEFFREY RUBNITZ AND SURESH SUBRAMANI*

Department of Biology, University of California, San Diego, La Jolla, California 92093

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Although DNA sequence homology is believed to be a prerequisite for homologous recombination events in procaryotes and eucaryotes, no systematic study has been done on the minimum amount of homology required for homologous recombination in mammalian cells. We have used simian virus 40-pBR322 hybrid plasmids constructed in vitro as substrates to quantitate intramolecular homologous recombination in cultured monkey cells. Excision of wild-type simian virus 40 DNA by homologous recombination was scored by the viral plaque assay. Using a series of plasmids containing 0 to 243 base pairs of homology, we have shown that the recombination frequency decreases as the homology is reduced, with the sharpest drop in recombination frequency occurring when the homology was reduced from 214 to 163 base pairs. However, low recombination frequencies were also observed with as little as 14 base pairs of homology.

Mammalian cells possess the ability to catalyze genetic recombination events, which may lead to such diverse genome rearrangements as sister chromatid exchange (16, 36), the excision of small circular DNAs from chromosomes (3, 25), gene amplification during acquisition of drug resistance (28), or gene rearrangements generating chromosomal deletions or inversions during the differentiation of immunoglobulin-producing cells (2, 27). Both homologous and nonhomologous recombination occur in cultured, mitotically dividing, mammalian cells, as shown by the formation of recombinant viruses after infection with genetically (5, 39, 41, 42) and physically (38, 44) distinguishable viruses or by the generation of infectious viral DNA with noninfectious DNA as the recombination substrate (13, 34, 38, 43). Homologous recombination has also been studied with vectors carrying overlapping gene fragments (26) or deletion and insertion mutations in selectable marker genes (17, 29, 31). But despite the accumulating evidence for homologous recombination, very little is known about the frequency, mechanism, or enzymology of this process in mammalian cells.

Much of our current knowledge about recombination mechanisms comes from studies in procaryotes (23) or various fungi (33). Essentially all proposed mechanisms for homologous recombination, like the Holliday model (11, 12), the Meselson-Radding model (21), and the double-strand-break repair model (35), involve the pairing of two different DNA molecules in a region of homology. According to these proposals, sequence homology is required for both the formation of recombination intermediates and subsequent branch migration.

Sequence homology is also important in a number of procaryotic and eucaryotic systems for recombination between nonhomologous DNA segments. These recombination events involve very short regions of homology, often at specific sites. For example, the inversions catalyzed by the Hin protein during phase variation in *Salmonella* spp. are generated by recombination between inverted repeats (45). A number of related procaryotic proteins, like Gin from phage Mu (7), Cin from phage P1 (14, 22), and TnpR from Tn*3* (9, 24), also catalyze recombination events between specific sites containing small regions of homology. The integration and excision of lambdoid phages (15) and the recombination events seen with the phage P1 lox-cre system (10) are also mediated through short homologous sequences. In eucaryotes, the FLP protein of the yeast 2μ plasmid catalyzes site-specific recombination between inverted repeats present on the plasmid (4). Other demonstrations of the importance of short homologies include nonhomologous recombinations lacking site specificity. Nonhomologous recombination between phage λ DNA and pBR322 DNA in *Escherichia coli* may involve 10 to 13 base pairs (bp) of homology (18), whereas deletions in *E. coli* may be formed by recombination between homologous regions of 6 to 17 bp (1).

Because sequence homology is such an important feature of recombination, several investigators have studied the effects of decreasing the length of homology available for specific recombination events. First, Singer et al. (30) determined that a minimum of 50 bp of homology is required by the major recombination pathway of bacteriophage T4. More recently, Gonda and Radding (8) found that *E. coli* RecA protein efficiently paired molecules that shared 151 bp of homology but failed to pair molecules in which homology was limited to 30 bp. In the present work, we have used previously described pBR322-simian virus 40 (SV40) hybrid plasmids (34) to investigate the minimum amount of homology required for recombination in monkey cells. After transfection of these plasmids into permissive cells, homologous recombination events leading to the formation of infectious SV40 virions can be easily scored by a plaque assay (see below). By progressively decreasing the length of homology from 5,243 to 0 bp, we have measured recombination frequency as a function of the length of homology. Our results show that the steepest drop in recombination frequency occurred between 163 and 214 bp, with lower levels of recombination occurring when there was only 14 bp of homology.

MATERIALS AND METHODS

Enzymes and linkers. Restriction endonucleases, exonuclease Bal-31, T4 DNA ligase, *E. coli* DNA polymerase I,

* Corresponding author.

and polymerase I Klenow fragment were purchased from Bethesda Research Laboratories or New England Biolabs and used as recommended. Oligonucleotide linkers were from New England Biolabs.

Animal cells and viruses. Conditions for the growth of CV1P monkey cells in 5% newborn calf serum and propagation of wild-type SV40 have been described previously (34).

Substrates for recombination. The substrates used to measure recombination frequencies were derivatives of plasmid pBSVD237, described earlier (34) (Fig. 1). pBSVD237 is a pBR322-SV40 plasmid containing a complete, intact copy of SV40 DNA and a duplication of the DNA segment between the SV40 *Bam*HI (map position, 0.14) and *Bcl*I (map position, 0.189) restriction enzyme sites. Because the duplicated segments are present in a direct (head-to-tail) configuration, any recombination event within the duplication yields a wild-type SV40 genome as one of the products.

To decrease the length of homology in the substrates, pBSVD237 was first digested partially with *Bam*HI to obtain full-length linear DNA. The DNA ends were made blunt by treatment with *E. coli* DNA polymerase I (Klenow fragment) and dNTPs, ligated to synthetic *Xho*I linkers (CCTCGAGG), digested with *Xho*I, and circularized by ligation with T4 DNA ligase. After transformation of *E. coli* HB101 with this ligation mixture, followed by restriction endonuclease analysis of the plasmid DNAs in several ampicillin-resistant colonies, the desired plasmid (pBSVD237X, Fig. 2A) was identified. Twenty-eight micrograms of pBSVD237X was then digested to completion with *Xho*I and treated with 5.6 U of exonuclease Bal-31 for 10 to 60 min at 15°C. The DNA samples from these time points were pooled, and their ends were again made blunt, ligated to *Xho*I linkers, cut with *Xho*I, circularized, and used to transform strain HB101. Plasmids ΔA through ΔG were identified among the amp^r colonies and chosen for further study. Each of these plasmids (except ΔG, see below) contains a complete SV40 genome, a duplication of less than 237 bp, and a unique *Xho*I site.

Plasmid pBSV2A (34) also was used in this study as a control plasmid containing only 4 bp of homology. It contains exactly one genome length of SV40 inserted at the *Bam*HI site of pBR322 (Fig. 2B).

Quantitation of recombination frequencies. The pBSVD plasmids were transfected into CV1P cells with DEAE-dextran (20). Wild-type SV40 genomes, formed by homologous recombination, were scored as viable virus by plaque assay (20). The number of plaques produced by transfection with a given pBSVD plasmid was normalized to the value obtained in parallel infections with wild-type SV40 DNA. The specific infectivity (PFU per microgram) for each DNA was obtained from the slopes of linear plots of PFU versus amounts of SV40 DNA for each of the transfecting DNAs. The slopes were obtained by a linear regression analysis.

DNA sequencing. To determine the exact lengths of homology, plasmids ΔA through ΔG were digested with *Xho*I and labeled at both ends with [γ-^{32}P]ATP and polynucleotide kinase. Fragments labeled at only one end were obtained for sequencing by digestion with a second restriction enzyme and purification of the appropriate DNA fragment from agarose gels by the glass powder method (40). The DNA sequence across each *Xho*I linker-SV40 DNA junction was then determined by the Maxam and Gilbert procedure (19).

RESULTS

Homologous recombination assay. Plasmid pBSVD (Fig. 1) contains intact SV40 early and late regions and can therefore replicate and synthesize all viral gene products after transfection into permissive cells, but because this plasmid is too large to be packaged in virions, mature virus and plaques cannot be produced. This plasmid, however, also contains a duplicated segment of SV40, providing a region of intramolecular homology. Because the homologous regions are present in a direct configuration, recombination within these regions yields two products (Fig. 1). One product is a wild-type SV40 genome that can be scored as a viable virus by a plaque assay. The other product, consisting of pBR322 DNA and a small segment of SV40, cannot replicate in mammalian cells and is not detected by this assay.

The plaque assay was chosen to measure recombination frequency primarily because of its high sensitivity. For example, since SV40 DNA has a specific infectivity of ca. 3.6×10^6 PFU/μg (34), a specific infectivity, or recombination frequency, of only 0.001% would still produce plaques at a measurable rate. A second advantage of the plaque assay is that each plaque represents an independent recombination event. Thus, individual recombination products can be easily isolated, propagated, and analyzed.

The SV40 plaque assay has been previously used to

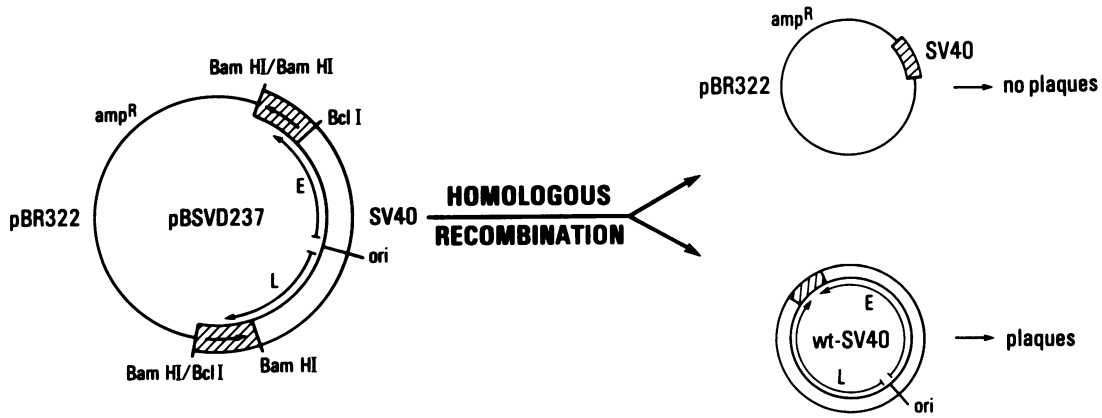

FIG. 1. Substrates for homologous recombination. DNAs of the pBSVD series contain more than one genome equivalent of SV40 DNA (double arcs) inserted at the *Bam*HI site of pBR322 (single arcs). Each pBSVD plasmid contains a head-to-tail, nontandem duplication (hatched region). The SV40 early region (E), late region (L), and origin of replication (ori) are shown. Homologous recombination within the hatched region yields two products, one of which is wild-type SV40 DNA and is thus capable of producing plaques.

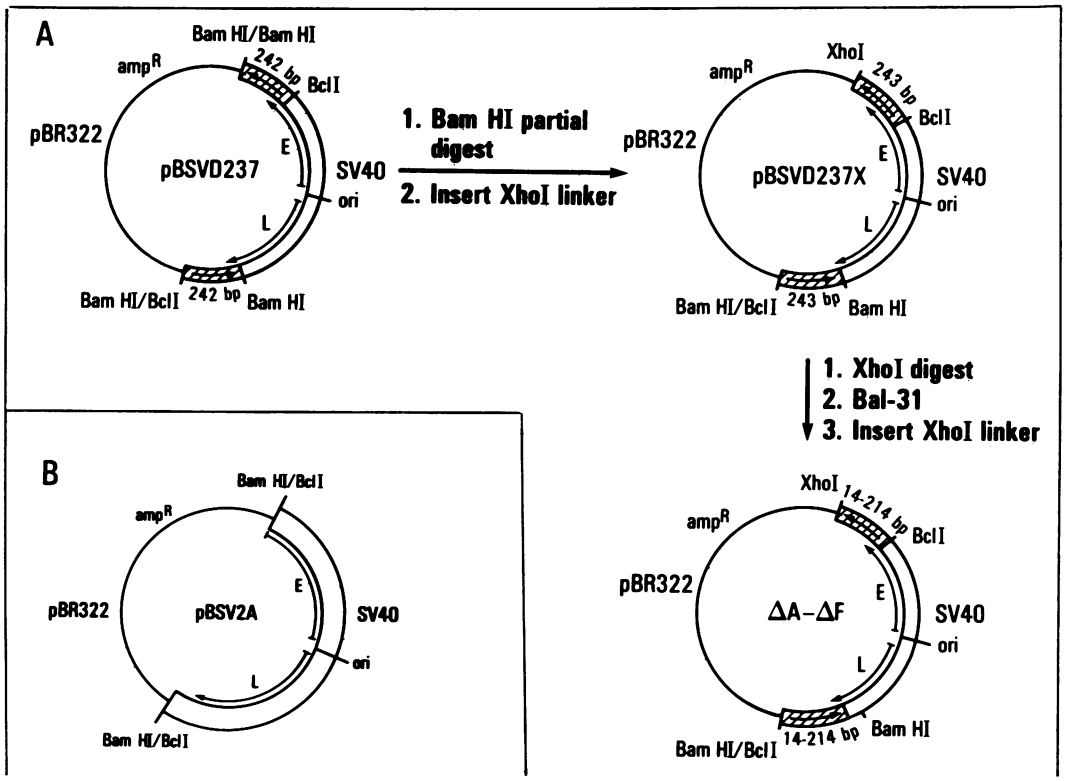

FIG. 2. Recombination substrates with decreasing amounts of homology. (A) pBSVD237, containing 242 bp of homology, was first converted to pBSVD237X, with 243 bp of homology as indicated. pBSVD237X was treated with Bal-31 as described in the text to create plasmids ΔA through ΔF, containing 14 to 214 bp of homology. (B) pBSV2A contains one genome length of SV40 DNA inserted at the *Bam*HI site of pBR322. The SV40 DNA is interrupted at the *Bcl*I site of the early region.

measure recombination frequencies with pBSVD plasmids (34). Transfections of CV1P cells with pBSVD237, pBSVD943, and pBSVD5243 (containing 242, 943, and 5,243 bp of homology) produced plaques at frequencies of 0.3, 2.7, and 13.4% of wild-type SV40 DNA. Furthermore, gel-purified supercoiled pBSVD DNAs produced plaques at frequencies similar to those cited above, indicating that the recombination was, in fact, occurring in the CV1P cells. Finally, independent recombination products were analyzed and shown to be identical to SV40 DNA (34).

Recombination substrates with decreasing amounts of homology. In the present study we have measured recombination frequency as a function of decreasing length of homology by using the plaque assay with derivatives of pBSVD237 (Fig. 2A). pBSVD237 was first converted to pBSVD237X by insertion of an *Xho*I linker at one *Bam*HI site, as shown. pBSVD237X was then digested with *Xho*I and treated with exonuclease Bal-31 to create deletions in the duplicated segment of SV40. Plasmids ΔA through ΔG, each containing a complete SV40 genome (except ΔG) and a duplication of less than 237 bp, were chosen for further study.

Because plasmids ΔA through ΔG were constructed with exonuclease Bal-31, it was necessary to determine the exact length of homology for each one. The unique *Xho*I site at the end of the duplicated SV40 segment provided a convenient site from which to sequence the plasmids. After digestion with *Xho*I and end labeling with [γ-^{32}P]ATP, the *Xho*I linker-SV40 DNA junction fragments of ΔA through ΔG were isolated and sequenced by the Maxam and Gilbert procedure (19).

pBSVD237X, which was not treated with Bal-31, has 243 bp of homology, extending from SV40 nucleotide 2532 through nucleotide 2774 (Table 1). The Bal-31 deletions, however, have homologies ranging from 14 bp in ΔF to 214 bp in ΔA. Only ΔG has no duplication and actually contains less than one genome length of SV40. It should be noted that as a consequence of the treatment of pBSVD237X with Bal-31, plasmids ΔD to ΔG probably cannot replicate (before recombination) in CV1P cells. In ΔD, the SV40 early region polyadenylation signals (6) have been deleted, but the large-T-antigen coding region is intact, whereas in plasmids ΔE to

TABLE 1. Lengths of homology in pBSVD plasmids

Plasmid	Sequence across *Xho*I linker[a]-SV40 junction	Length of homology (bp)
	2534[b]	
pBSVD237X	CCTCGAGG/GATCCA	243
	2562	
ΔA	CCTCGAGG/AGTTTG	214
	2578	
ΔB	CCTCGAGG/AACTAG	197
	2613	
ΔC	CCTCGAGG/AAATTT	163
	2682	
ΔD	CCTCGAGG/GCATTC	93
	2721	
ΔE	CCTCGAGG/AGGTTT	56
	2761	
ΔF	CCTCGAGG/GGCTGA	14
	2780	
ΔG	CCTCGAGG/CAGACT	0

[a] *Xho*I linker is CCTCGAGG.

[b] Nucleotide number of first SV40 nucleotide ligated to *Xho*I linker. The various plasmids with decreasing amount of homology were made as described in the legend to Fig. 2A.

ΔG both the polyadenylation signals and a portion of the large-T-antigen coding sequence are deleted. The control plasmid pBSV2A, although not sequenced here, has 4 bp of homology (nucleotides 2771 through 2774). This plasmid rarely produces plaques upon transfection of CV1P cells and gives only nonhomologous recombination products that are deletion or substitution mutants of SV40 (34).

Recombination frequencies. Recombination frequencies for pBSVD237X and its derivatives were determined by measuring plaque formation after transfection into CV1P cells. The specific infectivities of these plasmids, normalized as the percentage of SV40 DNA infectivity, decrease from 0.306% for pBSVD237X to 0.002% for ΔF (Table 2). For the entire series of plasmids, recombination frequency decreases each time the length of homology is decreased. Recombination frequency is proportional to length of homology over two separate ranges (Fig. 3). Recombination frequency first decreases linearly as the length of homology decreases from 5,243 to 214 bp and again decreases linearly from 163 to 14 bp. Between 214 and 163 bp, however, there is a ninefold decrease in frequency of recombination.

The recombination products are not generated in bacteria. Two lines of evidence indicate that the recombination events generating wild-type SV40 DNA occurred in the CV1P cells rather than in the recombination-deficient bacteria in which the substrates were grown. Southern blot analysis (32) confirmed that the pBSVD plasmid preparations contained no wild-type SV40. Five micrograms of each hybrid plasmid (more than 50 times the amount used in the plaque assays) was electrophoresed on an agarose gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled SV40 DNA. None of the pBSVD plasmids showed any hybridizing DNA the size of SV40 under conditions in which 10 pg of SV40 marker DNA was easily detected. Furthermore, each of the pBSVD plasmids was purified by gel electrophoresis and used to transfect CV1P cells. The frequency of plaque formation with gel-purified plasmid was unchanged.

Nature of the recombination products. For all plasmids studied, viruses from independent plaques were cloned and virus stocks and DNA were prepared. Approximately 83% of the isolates produced DNA that was indistinguishable from wild-type SV40 DNA with respect to size and pattern of digestion with a number of different restriction enzymes. The plaques that produced aberrant forms (e.g., deletions) of SV40 were distributed among infections with the different plasmids. Thus, it seems likely that most plaques arose from homologous recombination between the duplicated segments of SV40 DNA.

TABLE 2. SV40 plaque assays as a measure of recombination frequency[a]

DNA used in transfection	Homology (bp)	Specific infectivity (PFU/μg of DNA)	Specific infectivity normalized as % SV40 DNA
SV40		1.56×10^6	100
pBSVD237X	243	4.78×10^3	0.306
ΔA	214	3.87×10^3	0.248
ΔB	197	1.56×10^3	0.099
ΔC	163	4.27×10^2	0.027
ΔD	93	1.48×10^2	0.010
ΔE	56	57	0.004
ΔF	14	39	0.002
pBSV2A	4	<10	<0.001
ΔG	0		<0.001

[a] CV1P cells were transfected with the listed DNAs by the DEAE-dextran procedure (20). Plaques were scored after 8 to 12 days at 37°C. The values represent average measurements, using at least four DNA concentrations repeated three to five times each. The specific infectivities (column 3) for each assay were within 15% of the average values cited above.

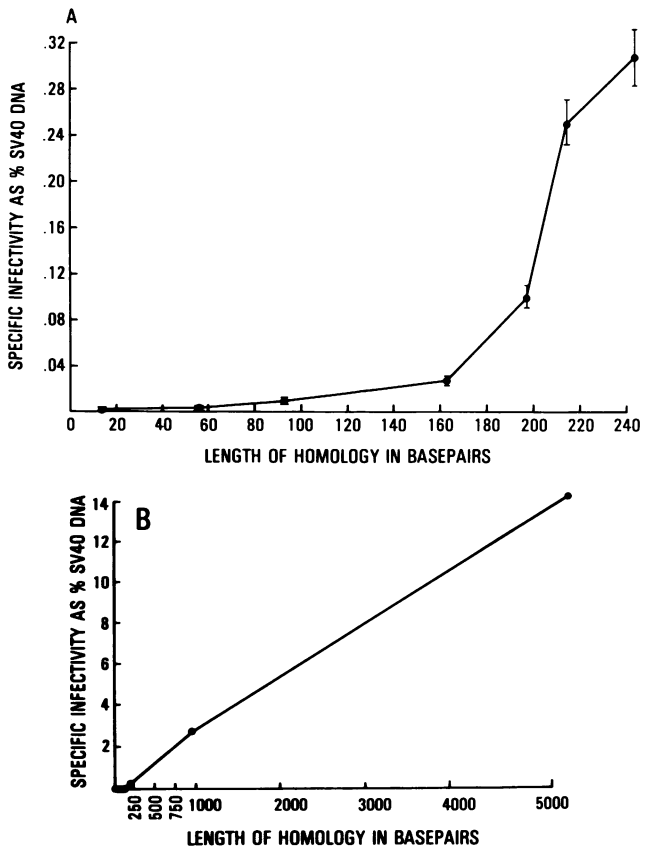

FIG. 3. Recombination frequency versus length of homology. (A) Recombination frequencies, expressed as specific infectivities (Table 2), are plotted as a function of length of homology for plasmids ΔF (14 bp of homology) through pBSVD237X (243 bp of homology). (B) Data for pBSVD943 and pBSVD5243 (943 and 5,243 bp of homology) from Subramani and Berg (34).

DISCUSSION

Using the SV40 plaque assay to quantitate homologous recombination in monkey cells, we measured recombination frequency as a function of length of homology. Intramolecular homologous recombination decreases linearly as length of homology is progressively decreased from 5,243 to 214 bp but decreases more rapidly thereafter, with a ninefold drop in the recombination frequency between 214 and 163 bp. Our results thus indicate that ca. 200 bp of homology are required for efficient recombination in monkey cells. Below this threshold, homologous recombination still occurs at a low but detectable rate. It appears, then, that there may be two pathways of recombination in mammalian cells, as has been proposed by Singer et al. for bacteriophage T4 (30). In T4, one pathway of recombination catalyzes recombination only between DNA segments sharing more than 50 bp of homology, whereas a second, less efficient pathway requires less homology. In mammalian cells, one pathway requires more than 200 bp of homology, with the second pathway requiring less. The simplest interpretation of the data would suggest that this minimum homology reflects the size of the site with

which the recombination enzymes interact in a stable fashion.

The drop in recombination frequency between 214 and 163 bp of homology is also interesting in view of the recent finding that homologous pairing by *E. coli* RecA protein requires between 30 and 151 bp of homology (8). It might be speculated that one, or several, of the proteins involved in recombination in mammalian cells requires ca. 200 bp of homology. Furthermore, Thomas (37) and Gonda and Radding (8) propose that a minimum recognition length for homologous pairing may explain how the enzymatic machinery of homologous recombination guards against mistakes. According to this model, an organism with a larger genome would be expected to have a larger minimum recognition length. Our results, indicating a minimum length of 200 bp of homology for mammalian cells compared with 50 bp of homology for T4 and 30 to 151 bp for *E. coli*, are thus consistent with the model.

Earlier studies (34) have shown that neither replication of the pBSVD plasmids nor the expression of large T antigen is required for homologous recombination and that the plasmids that can replicate show only a twofold higher recombination frequency than plasmids with the same amount of homology that cannot replicate. In this study, ΔA to ΔC (214 to 163 bp of homology) have complete early regions (i.e., large-T-antigen coding region and polyadenylation signals) and should therefore replicate with equal efficiency before recombination. Thus, the drop in recombination frequency corresponding to a decrease in homology from 214 to 163 bp is not related to differences in the ability of plasmids ΔA to ΔC to replicate.

Despite the sharp cutoff in recombination frequency between 214 and 163 bp, we have detected homologous recombination even in plasmids where homology was limited to only 14 bp. The ability of mammalian cells to catalyze recombination events between such small homologies is not surprising and might be analogous to deletion formation between small random regions of homology in bacteria (1).

Finally, several features of our recombination assay should be emphasized. Because we measured recombination events that occurred at low frequencies, it was necessary to ensure that the events actually occurred after transfection of the plasmids into CV1P cells, rather than during their propagation in recombination-deficient *E. coli*. Southern blot analysis (32) and plaque assays with gel-purified plasmids confirmed that wild-type SV40 DNA was absent from our substrates and was generated in the CV1P cells, presumably through homologous recombination. Although our recombination assay allows us to detect only intramolecular recombination between DNA segments that are ca. 5 kilobases apart, we presume that the amount of homology essential for stable synaptic or postsynaptic events will be the same for intermolecular recombination or intramolecular recombination between homologous segments separated by different distances. Our results thus suggest that the enzymatic machinery catalyzing extrachromosomal recombination in mammalian cells requires ca. 200 bp of homology.

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