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Homologous and Nonhomologous Recombination in Monkey Cells

SURESH SUBRAMANI† AND PAUL BERG*

Department of Biochemistry, Stanford University Medical Center, Stanford, California 94305

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Though recombinational events are important for the proper functioning of most cells, little is known about the frequency and mechanisms of recombination in mammalian cells. We have used simian virus 40 (SV40)-pBR322 hybrid plasmids constructed in vitro as substrates to detect and quantitate intramolecular homologous and nonhomologous recombination events in cultured monkey cells. Excision of wild-type or defective SV40 DNAs by recombination from these plasmids was scored by the viral plaque assay, in either the absence or the presence of DNA from a temperature-sensitive helper virus. Several independent products of homologous and nonhomologous recombination have been isolated and characterized at the DNA sequence level. We find that neither DNA replication of the recombination substrate nor SV40 large T antigen is essential for either homologous or nonhomologous recombination involving viral or pBR322 sequences.

A common feature of most existing models for genetic recombination is the formation of Holliday-type intermediates (18, 30); these contain heteroduplex helices formed by homologous base pairing of strands from two different segments of DNA (18, 29). Much of the supporting evidence for the involvement of such structures in genetic recombination derives from studies with bacterial and bacteriophage mutants blocked in recombination (6, 37), physical characterization of the structure of the putative recombination intermediates (10, 21, 29, 44), and the analysis of the bacterial enzymatic machinery involved in the recombination process (8, 9, 17, 30). Genetic recombination also occurs in mammalian somatic cells, as evidenced by the formation of recombinant viruses after infection of cultured cells with genetically (11, 47, 48) and physically (46, 52) distinguishable viruses. Homologous recombinants have also been detected and studied in transfections with specially designed DNA molecules that yield infectious virus from noninfectious precursors (19, 46, 50, 51). Although it is generally assumed that the pathways of genetic recombination in procaryotes and eucaryotes are similar, if not identical, very little has been done to explore the intermediates, mechanisms, and enzymology of this process in mammalian cells.

Genetic recombination also occurs between nonhomologous DNA segments, often at specific sites. Sometimes, such recombinations involve short regions of homology, such as in the integration of lambdoid phage (23), in the gene rearrangements during differentiation of immunoglobulin-producing cells (4, 34), and the transpositions that occur in phase variations of *Salmonella* (53) and mating type changes of *Saccharomyces* (16, 41). Nonhomologous recombinations, apparently lacking site specificity, have been implicated in the integration of virus or exogenous DNAs into cellular chromosomes (1, 26, 35), the excision of integrated viral DNA after fusion of permissive and simian virus 40 (SV40)-transformed cells (2, 3, 14, 22, 49), the circularization of linear DNA (28, 36), and the creation of deletions (7, 28), substitutions (5, 33, 45), and some duplications (20, 45) during replication of viral DNA. Some models have been proposed to explain certain site-specific recombinations (23, 34, 40, 53), but the molecular basis of general, nonhomologous recombination is an enigma (39).

Several problems complicate the analysis of nonhomologous recombination. One is the paucity of assays to score the event; another is the difficulty in identifying the partners and the products of the nonhomologous recombination event. In the present work, we have used a relatively simple approach to detect the occurrence and identify the products of intramolecular nonhomologous recombinations; the assay also provides a semiquantitative comparison of the probability of nonhomologous and homologous recombination within a common substrate.

† Present address: Department of Biology, University of California, San Diego, La Jolla, CA 92093.

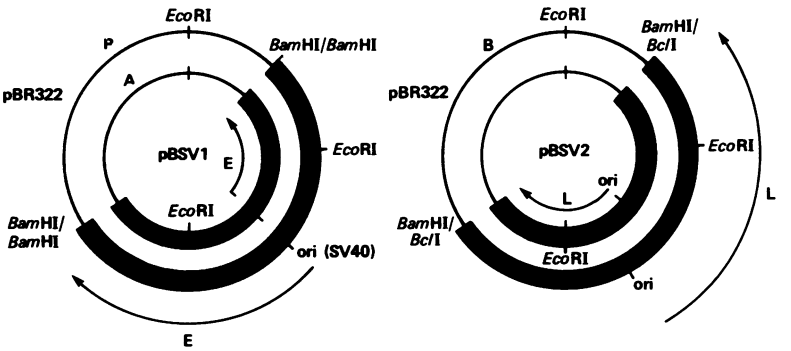

FIG. 1. Substrates used to detect nonhomologous recombination. Molecules in the pBSV series contain one genome length of SV40 DNA (solid arcs) inserted at the *Bam*HI site of pBR322 DNA (thin arcs), as described in the text. For either pBSV1, in which the SV40 DNA is interrupted at the *Bam*HI site of the late region, or pBSV2, which has the SV40 DNA interrupted at the *Bcl*I site of the early region, there are two orientations of the SV40 segment relative to pBR322 sequences. Thus, recombinants pBSV1A and pBSV1B are shown on the inner and outer circles, respectively; pBSV2A and pBSV2B are shown in the same format.

The experimental protocol involves measurements of the frequency of production of infectious SV40 virions (containing wild-type and defective genomes) after transfection of a permissive host with pBR322-SV40 hybrid plasmids that contain either a single SV40 genome length (Fig. 1) or a single genome length plus increasing lengths of SV40 DNA (Fig. 2). Our data indicate that the probability of intramolecular homologous recombination is higher the greater the length of the duplicated sequence homology. Moreover, we find that the recombination event is not dependent on replication of the DNA substrate. Our experiments also reveal that in-

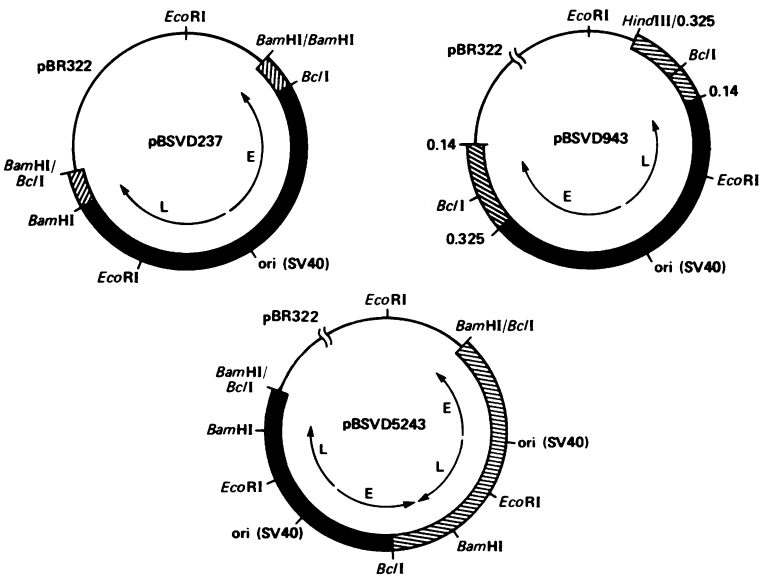

FIG. 2. Substrates used to detect homologous recombination. DNAs of the pBSVD series contain more than one genome equivalent of SV40 DNA cloned in pBR322. Each recombinant contains a head-to-tail nontandem duplication (hatched area) whose length (in bp) is indicated by the number after pBSVD (e.g., pBSVD237). Early (E) and late (L) regions in the virus DNA are marked on the insides of the circles. The SV40 origin of DNA replication (ori) and recognition sites for some restriction endonucleases are shown. The numbers 0.325 and 0.14 in pBSVD943 refer to map positions on the SV40 genome.

tramolecular nonhomologous recombination is frequent, occurring at least once in 5 to 10% of the transfected cells. Here, too, nonhomologous recombination occurs even if the DNA substrate cannot replicate before the recombination event. Moreover, both the homologous and nonhomologous recombination events are probably mediated by host cell enzymes, since none of the SV40 gene products are essential for recombination.

MATERIALS AND METHODS

Enzymes. Restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs and were used as recommended by the purveyors. T4 DNA ligase and *Escherichia coli* PolI were gifts of S. Scherer, Stanford University, and S1 nuclease was from Boehringer Mannheim.

Animal cells and viruses. Conditions for the growth of CV1 monkey cells and the procedure for propagation of SV40 mutants with an excess (10 ng per 60-mm plate) of helper DNAs have been described by Mertz and Berg (27), except that cells were grown in 5% newborn rather than fetal calf serum. Newborn calf serum was from Microbiological Associates.

Substrates for recombination. The substrates for recombination were hybrid plasmids of pBR322 and SV40 DNA. Substrates of one type, the pBSV plasmids (Fig. 1), contain one copy of SV40 DNA inserted into the *Bam*HI restriction site of pBR322 DNA. They were prepared and cloned by procedures analogous to those described below. In one set of recombinants (pBSV1), the viral DNA was interrupted in the late region at the *Bam*HI restriction site (map position 0.14), and in the other (pBSV2), the interruption of the SV40 DNA sequence occurred in the *Bcl*I restriction site of the early region (map position 0.189). The plasmids pBSV1A or B and pBSV2A or B are the recombinants with the two possible orientations of the SV40 relative to pBR322 DNA (Fig. 1).

Another set of pBR322-SV40 hybrid plasmids contained, in addition to one SV40 DNA length, duplicate segments of SV40 DNA ranging in size from 237 base pairs (bp) (pBSVD237) to 5,243 bp (pBSVD5243) (see Fig. 2).

pBSVD237. pBSVD237 contained a complete copy of SV40 DNA and a duplication of the segment between the *Bam*HI and *Bcl*I restriction sites of SV40. The 237-bp fragment was inserted in the *Bam*HI site of pBR322, and the resulting plasmid (pBRSV11) was identified (after transformation of HB101) among the ampicillin-resistant (Amp^r), tetracycline-sensitive (Tet^s) colonies. Then linear SV40 DNA, cleaved with *Bam*HI endonuclease, was inserted at the unique *Bam*HI restriction site of pBRSV11 and cloned in HB101. Amp^r colonies were hybridized with a ^{32}P-labeled fragment of SV40 DNA from the region between map positions 0.65 and 0.86 by the Grunstein and Hogness procedure (15). The structure expected for pBSVD237 (Fig. 2) was confirmed by analyses of the plasmid DNA with appropriate restriction enzymes (data not shown).

pBSVD943. pBSVD943 contained a 943-bp duplication of SV40 DNA from the *Hin*dIII restriction site at map position 0.326 to the *Bam*HI site at map position 0.144. The 943-bp *Hin*dIII-*Bam*HI fragment was first cloned between the *Hin*dIII and *Bam*HI restriction sites of pBR322 DNA (pBRSV1A). Then pBRSV1A DNA was digested with *Bam*HI endonuclease, the DNA ends were made blunt by treatment with S1 nuclease, and the DNA was circularized by blunt-end ligation with T4 DNA ligase (pBRSV1B). pBRSV1B DNA lacks the *Bam*HI restriction site but contains a unique *Bcl*I endonuclease cleavage site (in the SV40 insert) into which additional SV40 DNA can be inserted. Because the recognition sequence for *Bcl*I endonuclease (TGATCA) is modified by the DNA adenine methylase gene (dam) in HB101, pBRSV1B DNA had to be propagated in a *dam* mutant of *E. coli* (24) before it could be digested with *Bcl*I endonuclease. After digestion of the SV40 DNA with *Bcl*I endonuclease, it was ligated into the *Bcl*I site of pBRSV1B, and the resulting DNA was used to transform HB101 to Amp^r. Colonies harboring pBSVD943 plasmid DNA (Fig. 2) were identified by colony hybridization with the SV40 *Hin*dIII fragment derived from map position 0.65 to 0.86; their structures were confirmed by restriction enzyme mapping (data not shown). Only clones containing the SV40 DNA in the orientation shown in Fig. 2 were recovered; presumably, those containing the SV40 DNA in the opposite orientation were not obtained because plasmids with the 943-bp inverted repeat were selected against.

pBSVD5243. pBSVD5243 is a head-to-tail, tandem dimer of SV40 DNA cloned in pBR322. Linear SV40 DNA, obtained by digestion of supercoiled viral DNA with *Bcl*I endonuclease, was inserted at the *Bam*HI restriction site of pBR322. The joins of the resulting cloned plasmid DNA (pBSV2A in Fig. 1) were not cleaved with either *Bcl*I or *Bam*HI endonuclease, but pBSV DNA still contained a unique *Bam*HI restriction site within the SV40 DNA at map position 0.14. Another copy of SV40 DNA cleaved with *Bam*HI endonuclease was inserted at this *Bam*HI site to yield pBSVD5243 (Fig. 2). Again, only one arrangement of the two SV40 DNA segments was obtained.

Substrates to test the requirement for DNA replication and large T antigen in recombination. SV40 large T antigen is required for replication of SV40 viral DNA in permissive cells (45). To determine whether replication or large T antigen is essential for homologous or nonhomologous recombination, hybrid plasmids were constructed with interruptions of the large T antigen gene. As a consequence, the formation of large T antigen and DNA replication cannot occur until after recombination reconstitutes a functional large T antigen gene.

pBSVD1969. pBSVD1969 was the substrate for assessing the requirement of large T antigen for homologous recombination. It has a duplication of 1,969 bp of SV40 DNA (from the *Taq*I restriction site at map position 0.57 to the *Bcl*I site at map position 0.19). The 1,969-bp *Taq*I-*Bcl*I SV40 DNA fragment was cloned initially between the *Cla*I and *Bam*HI restriction sites of pBR322 DNA. This derivative, pBRSV8, has two *Pst*I restriction sites: one in the Amp gene segment and the other at map position 0.27 of the SV40 insert. SV40 DNA also contains two *Pst*I restriction sites; therefore, both pBRSV8 and SV40 DNA were digested partially with *Pst*I endonuclease to obtain full-length linear DNA. The DNAs were ligated together, and the ligation mixture was used to transform HB101

to Amp^r. The selection for Amp^r eliminates recombinants which have DNA inserted into the *Pst*I site of the Amp gene. Derivatives of pBRSV8 with SV40 DNA inserted into the other *Pst*I restriction site were identified by hybridization of the Amp^r colonies with the ^{32}P-labeled large *Taq*I-*Bcl*I fragment of SV40 DNA. Plasmid DNA obtained from positive colonies was analyzed by restriction enzyme digestions to identify pBSVD1969 (see Fig. 7). This molecule does not have an intact early region; therefore, it cannot replicate or make large T or small t antigens.

pNHR1. pNHR1 provided the substrate for nonhomologous recombination. The region between the *Bam*HI and *Bcl*I restriction sites in SV40 DNA contains the signals for early and late viral mRNA polyadenylation (13). Cloning of this 237-bp fragment into the *Bam*HI restriction site of pBR322 DNA yields the plasmid pBRSV11. Because pBRSV11 DNA has a unique *Cla*I cleavage site (in the pBR322 portion), linear pBRSV11 DNA, obtained by digestion with *Cla*I endonuclease, was ligated with linear SV40 DNA cleaved with *Taq*I endonuclease. The mixture was used to transform HB101 to Amp^r. The resulting colonies were hybridized with ^{32}P-labeled SV40 DNA that spanned the region from the *Taq*I to the *Bcl*I restriction sites. DNA having the structure and the orientation shown in Fig. 8A was confirmed by appropriate restriction enzyme digestions.

Quantitation of recombination frequencies. The pBSVD DNAs (Fig. 2), which contain various lengths of duplicated SV40 DNA segments, were transfected into CV1P cells with DEAE-dextran (27). These plasmid DNAs contain intact SV40 early and late regions and should express all viral gene products. However, since this DNA cannot be packaged in virions, mature virus (and plaques) cannot be produced. However, genomes of mature size can be formed by homologous recombination and, therefore, the recombinants can be scored as viable virus by plaque assay (27).

pBSV plasmids were also introduced into CV1P cells by the DEAE-dextran procedure. In this case, to reconstruct an intact late or early region, the recombination would have to have occurred at a specific base pair within the join; therefore, viable progeny were not expected from these transfections (see below). However, nonhomologous recombination could yield DNA molecules that are defective and cannot produce plaques alone, but which can be complemented by an appropriate helper genome. Thus, genomes with deletions of SV40 DNA or substitutions of pBR322 DNA for SV40 early and late regions segments were scored by plaque formation after coinfections of pBSV plasmids with appropriate *ts* mutant genomes (27).

In our measurements, the number of plaques produced by transfection with a given hybrid plasmid DNA and an excess of helper DNA 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 the amount of transfecting DNA, which was varied over 10- to 100-fold. Recombination frequencies are expressed as a percentage, calculated from the specific infectivity of the substrate DNA relative to that of wild-type SV40 DNA.

DNA sequencing. DNA fragments spanning the recombination junctions were isolated by appropriate restriction enzyme digestions and gel electrophoresis, and the DNA sequence across the recombination junction was determined by the Maxam and Gilbert procedure (25).

RESULTS

Several features and expectations for the behavior of the hybrid plasmid DNAs (Fig. 1 and 2) provide the rationale for the experimental plan. The single genome length of SV40 DNA in the pBSV plasmids is interrupted in the late (pBSV1) or early (pBSV2) region by the joins to pBR322 DNA. Therefore, after transfection of cells with these plasmids, wild-type virus genomes could be obtained only if recombination occurred precisely at the pBR322-SV40 DNA joins; in that case, the late and early regions would be reconstituted, and viral DNA would be reduced to packageable size. Because this recombination target is small, the probability of producing virus would be low, perhaps undetectable. On the other hand, the pBSVD hybrid plasmids (Fig. 2) have intact late and early regions and, therefore, can synthesize all of the viral gene products and replicate after transfection into cells; but recombination is essential to produce SV40 DNA of wild-type size. However, the duplicated segments of SV40 DNA in these plasmids provide intramolecular homology, and recombination at any site within the duplication can yield wild-type viral genomes.

Homologous recombination. The specific infectivities (PFU per microgram of DNA) after transfection of CV1P cells with pBSV and pBSVD type plasmids are summarized in Table 1. The specific infectivity for the pBSV plasmids is less than 0.0001% of that of wild-type SV40 DNA (set as 100%). In contrast, transfections with pBSVD237, pBSVD943, or pBSVD5243 plasmids produced plaques at frequencies of 0.3, 2.7, and 13.4%, respectively, of wild-type DNA.

Viruses from several independently arising plaques were cloned, and virus stocks and DNA were prepared from each. Each isolate yielded DNA that was indistinguishable from wild-type SV40 DNA with respect to size and restriction patterns with several different restriction endonucleases (data not shown).

Although unlikely, it was possible that the transfecting plasmid DNAs contained wild-type SV40 DNA, introduced inadvertently or produced by recombination in *E. coli*. Therefore, each of the pBSVD DNAs was purified by gel electrophoresis, and the band corresponding to the hybrid-sized DNA was recovered and used to transfect CV1P cells. The frequency of plaque formation with each DNA was, within experimental limits, unchanged.

These data indicate that wild-type SV40 DNA can be derived from the pBSVD-type hybrid plasmids. This contrasts with the result obtained

TABLE 1. SV40 plaque assays as a measure of recombinational frequency[a]

DNA used in transfection	Specific infectivity (PFU/μg of DNA) normalized as % of SV40 DNA
SV40	100
pBSV1A	0
pBSV1A + *tsA58* (excess)	5.4
pBSV1A + *tsB201* (excess)	0
pBSV1B	0
pBSV1B + *tsA58* (excess)	7.1
pBSV1B + *tsB201* (excess)	0
pBSV2A	0
pBSV2A + *tsA58* (excess)	0
pBSV2A + *tsB201* (excess)	2.9
pBSV2B	0
pBSV2B + *tsA58* (excess)	0
pBSV2B + *tsB201* (excess)	3.1
tsA58	0
tsB201	0
tsA58 + *tsB201* (excess)	50
tsB201 + *tsA58* (excess)	100
pBSVD237	0.3
pBSVD943	2.7
pBSVD5243	13.4
pBSVD237 + *tsA58* (excess)	6.9
pBSVD237 + *tsB201* (excess)	2.4
pBSVD943 + *tsA58* (excess)	7.4
pBSVD943 + *tsB201* (excess)	3.0
pBSVD1969	2
pBSVD1969 + *tsB201* (excess)	5.5
pNHR1	0.13
pNHR1 + *tsB201* (excess)	2.7

[a] CV1P cells were transfected with the listed DNAs by the DEAE-dextran procedure described in the text. Plaques were scored after 10 to 12 days at 37°C and after 12 to 15 days at 41°C. Infections with *tsA58* and *tsB201* were carried out at the nonpermissive temperature of 41°C. The values given represent the average measurements, using at least three DNA concentrations—each repeated two to four times—and are accurate to ±15% of the value cited. The average specific transfectivity of SV40 DNA was 3.6×10^6 PFU/μg. In any one set of transfections, each value was normalized to the value (100%) obtained with SV40 DNA.

with the pBSV plasmids which lack the duplication segments. Considering the structure and growth behavior of the recovered viruses, we infer that the wild-type genomes most probably originate by recombinational excision from the transfecting DNA, or by recombination from replicating forms. Whatever the source or mechanism, the larger the duplication, the greater the probability of producing recombinants.

Nonhomologous recombination. The pBSV-type hybrid plasmids, which contain only one genome equivalent of SV40 DNA cloned in the pBR322 plasmid (Fig. 1), provided substrates for studying nonhomologous recombination. Transfections of CV1P cells with pBSV hybrid plasmids did not yield infectious virus progeny (Table 1). But numerous infectious centers were produced if CV1P cells were cotransfected with either pBSV1A or pBSV1B and *tsA58* DNAs or with either pBSV2A or pBSV2B and *tsB201* DNAs at the nonpermissive temperature (41°C) (Table 1). Note that plaques were not produced in transfections of the same cells with the *ts* helper DNAs alone. Moreover, no plaques were obtained when the transfections were performed with either pBSV1A or B and *tsB201* DNA or when pBSV2A or B DNAs and *tsA58* DNA were used (Table 1). Therefore, we conclude that the infectious centers arise by complementation of the respective *ts* mutant genomes by defective progeny originating from the pBSV plasmids.

The relative specific infectivities of the plasmids interrupted in the late region (pBSV1A and B) were not significantly different (5.4 and 7.1%) for the two orientations of the SV40 and pBR322 DNA sequences. Somewhat lower values (2.9 and 3.1%) were observed consistently with plasmids interrupted in the early region (pBSV2A and B). These lower values probably stem from the lower efficiency of complementation when *tsB201* is used in excess for complementation (Table 1).

Structure of the recombinant genomes produced from pBSV plasmids. Viruses obtained from plaques generated with pBSV1 and pBSV2 (and the appropriate helper DNAs) were cloned by plaque purification, and high-titer stocks and DNA were prepared from the cloned viruses. Virus obtained from independent plaques contains, beside helper DNA, a single species of DNA that is physically distinct from wild-type DNA. As documented below, each new species is a deletion mutant or contains pBR322 DNA in place of either late or early region SV40 DNA sequences.

Digestion of *tsA58* DNA with *Pst*I yields two fragments 4,027 and 1,216 bp in length. When DNA obtained from a plaque produced by cotransfection with pBSV1A and *tsA58* DNA is digested with *Pst*I endonuclease, there is another DNA species (SVpBR8) which produces 4,027- and 890-bp fragments (Fig. 3A, track 1). This result indicates that SVpBR8 retains the two SV40 *Pst*I restriction sites but that one of the fragments has become smaller. Similarly, *tsA58* DNA is cut once by either *Hpa*II or *Taq*I endonuclease, but the same digestions of SVpBR8 DNA produce additional fragments, indicating that it contains more than one site for each of these enzymes. Moreover, the appearance of a 2.2-kb fragment after *Ava*II endonuclease digestion indicates that the SV40 *Ava*II site at nucleotide 2,013 is missing in SVpBR8 (Fig. 3A, track 2). Overall, the digests shown in Fig.

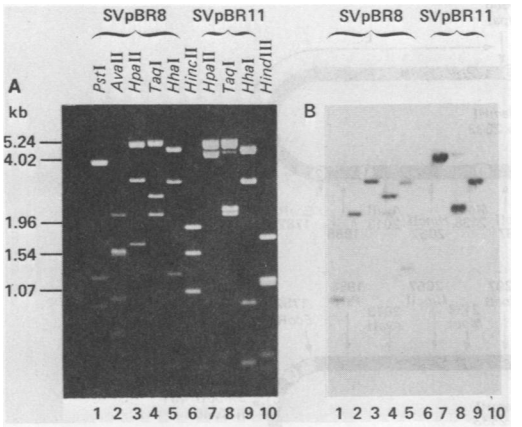

FIG. 3. Restriction endonuclease digests of two nonhomologous recombination products derived from pBSV1. SVpBR8 and SVpBR11 are two viral DNAs obtained from plaques in a mixed infection of CV1P cells with pBSV1A and *tsA*58 DNAs. (A) Tracks 1 to 5 contain digests of the mixture of SVpBR8 and *tsA*58 DNA with the enzymes indicated. Tracks 6 and 10 contain *Hin*cII and *Hin*dIII digests of SV40 DNA for size markers. Tracks 7 to 9 contain the indicated digests of the mixture of SVpBR11 and *tsA*58 DNA. (B) The DNAs shown in panel A were transferred to nitrocellulose, hybridized with nick translated pBR322 DNA, and autoradiographed (43). The numbers on the left provide size references.

3A demonstrate that SVpBR8 differs from SV40 DNA in having lost some SV40 restriction sites and acquired new ones.

Restriction enzyme digests of the DNA in another virus stock obtained from the same transfection confirmed that it too contained a DNA species smaller than wild-type SV40 (SVpBR11). Although SVpBR11 DNA retains the single *Hpa*II restriction site (Fig. 3A, track 7), it contains more than one *Taq*I and *Hha*I cleavage site (Fig. 3A, tracks 8 and 9). Thus, SVpBR11 DNA also differs significantly from SV40 DNA.

Support for the view that SVpBR8 and SVpBR11 are recombinant SV40 genomes containing pBR322 DNA segments in place of SV40 DNA was obtained by blotting the restriction digests shown in Fig. 3A to nitrocellulose (38) and hybridizing them with ^{32}P-labeled pBR322 DNA prepared by nick translation (32; Fig. 3B). Quite clearly, one or more fragments in each digest contained pBR322 DNA sequences. These data, combined with similar analyses of other restriction enzyme digestions, strongly indicate that SVpBR8 and SVpBR11 are derived from the hybrid plasmid pBSV1A by intramolecular recombination between pBR322 and SV40 DNA. Figures 4A, B, and C summarize the probable recombination sites for a collection of recombinant viral genomes. Thus, the recombination site inferred for SVpBR8 is shown by the number 1 in the pBR322 and SV40 DNA portions of pBSV1A (Fig. 4A), and SVpBR11 arises by recombination at the sites marked by the number 2. SVpBR7, on the other hand, results from an intramolecular recombination within the SV40 DNA sequence (Fig. 4A).

DNA sequences at the recombinant sites. Each of the isolates contained, in addition to the helper virus genome, a single recombinant DNA molecule. And, because the nucleotide sequences of SV40 and pBR322 DNA are known (12, 31, 42), the nucleotides involved at the recombination site can be defined precisely. Accordingly, ^{32}P-end-labeled fragments containing the nucleotide sequence of the putative recombination sites for the various recombinants were isolated and sequenced by the Maxam and Gilbert procedure (25). Figure 5 shows the nucleotide sequence ladders that identify where SV40 DNA sequences recombined to produce SVpBR7 and where the novel joints between SV40 and pBR322 DNA sequences occurred in SVpBR11 and SVpBR15B; these particular nucleotide sequences are shown in Fig. 6.

In SVpBR7, the sequence across the recombination junction is AAGCTG. This sequence would be generated either if SV40 nucleotide 2,631 were joined to nucleotide 2,183 or if nucleotides 2,630 and 2,182 were joined. Compared with SV40 DNA, the exact size of the deletion in SVpBR7 is 448 bp; this compares with the estimate of 450 bp from changes in the restriction fragment sizes in Fig. 3A. In SVpBR11, the C residue at SV40 nucleotide 1,757 is joined to a T residue at nucleotide 249 in pBR322; these sites also agree with those deduced from the data in Fig. 3A. The DNA of SVpBR15B has the T residue at nucleotide 1,780 of SV40 linked to the A residue at nucleotide 807 in pBR322; alternatively, nucleotide 1,781 of SV40 could be joined to nucleotide 806 in pBR322 DNA.

In these three instances, there is no apparent homology at or near the recombination site. A computer search (carried out by D. Brutlag) for regions of complete or partial homology within 100 nucleotides of the novel joint failed to detect any significant matches. We conclude, therefore, that the viral genomes recovered from transfections with the hybrid plasmids pBSV1A and B and pBSV2A and B in CV1P cells arise by nonhomologous recombination. Judging from these recombinants, there do not appear to be hot spots, nor is the frequency of recombination influenced by the particular nucleotide sequences (or region) within which the breakage and rejoining occur.

Comparison of homologous and nonhomolo-

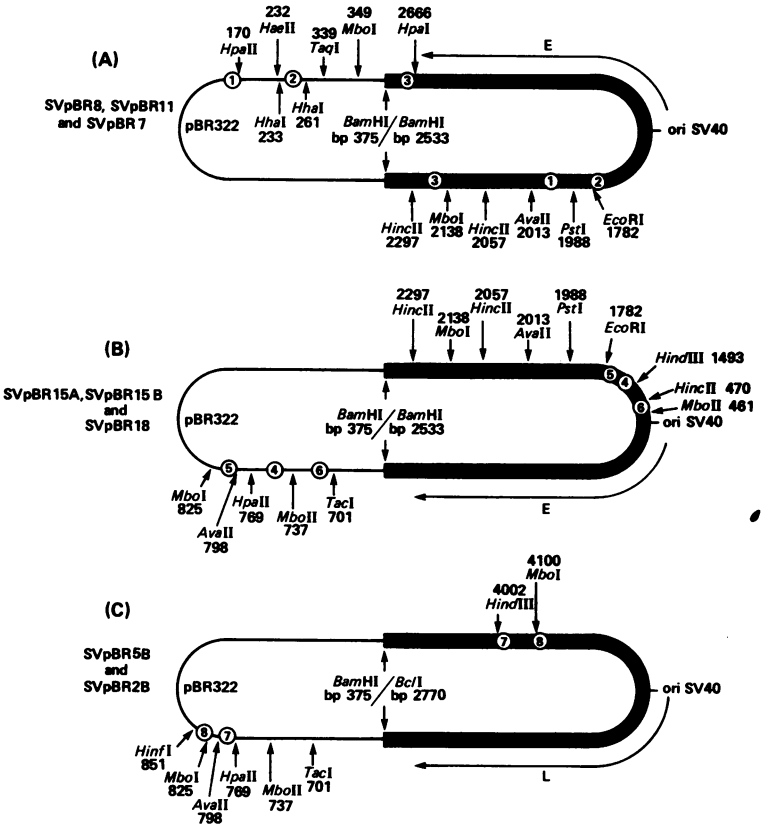

FIG. 4. Origins of several recombination products from transfections with pBSV substrates. (A), (B), and (C) show the structures of pBSV1A, pBSV1B, and pBSV2A, respectively, with SV40 indicated by the shaded portion. The sites for various restriction endonucleases and the appropriate nucleotide numbers from SV40 and pBR322 are indicated either above or below the arrows indicating the restriction sites. (A) Inferred recombination points for SVpBR8, SVpBR11, and SVpBR7 are indicated by the numbers 1, 2, and 3, respectively. (B) Recombination positions for SVpBR15A, SVpBR15B, and SVpBR18 are denoted by 4, 5, and 6, respectively. (C) Recombination points for SVpBR5B and SVpBR2B are numbered 7 and 8, respectively.

gous recombination in the same transfections. In a preceding section, we described the results of transfections with the pBSVD hybrid plasmids; these DNA molecules produce wild-type SV40 genomes, presumably via homologous recombination of duplicated segments of SV40 DNA. If cells are cotransfected with these plasmids and either *tsA*58 or *tsB*201 DNA, and the cells are maintained at 41°C, the specific infectivity of the DNA rises (Table 1). Thus, the specific infectivity of pBSVD237 DNA alone is 0.3% of that of wild-type SV40 DNA; but in the presence of excess *tsA*58 or *tsB*201 DNA, the values are 6.9 and 2.4%, respectively. Similarly, with pBSVD943, the comparable values were 2.7% without helper, 7.4% with *tsA*58, and 3% with *tsB*201. This result contrasts with the finding that transfections with pBSV1 plasmids yield plaques with *tsA*58 but not *tsB*201 as helper; and pBSV2 plasmids produce plaques with *tsB*201 but not *tsA*58 as helper. These observations imply that the pBSVD plasmids can participate in both homologous and nonhomologous recombination events; only the homologous recombinations are detectable in the absence of helper genomes, whereas both events are scored in the presence of helper. Because the pBSVD plasmids may undergo nonhomologous recombination involving either early or late region sequences, the ensuing products would require *tsB*201 or *tsA*58, respectively, as helper. Although they were not studied in detail, viruses from many of the plaques obtained from transfections with pBSVD237 or pBSVD943 in the presence of *tsA*58 or *tsB*201 contained deletions or substitutions of the type described above.

T antigens and DNA replication are not required for homologous or nonhomologous intramolecular recombination. Hybrid plasmids in which the SV40 early region is interrupted by

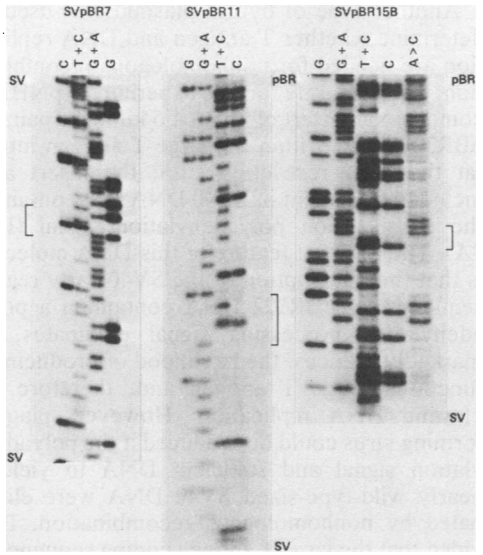

FIG. 5. DNA sequence ladders across the recombination junction in SVpBR7, SVpBR11, and SVpBR15B. The gels are read from the indicated SV40 nucleotides at the bottom across the recombination junction indicated by the brackets. The SV and pBR symbols indicate whether recombination occurred within SV40 or between SV40 and pBR322 sequences. The brackets indicate the sequence across the recombination joint. The sequences are shown in Fig. 6.

pBR322 DNA were used to investigate the requirement for T antigens and DNA replication for homologous and nonhomologous recombination. One of the plasmids contained segments of the same 1,969 bp of SV40 DNA at the two SV40-pBR322 boundaries (pBSVD1969 [Fig. 7]). With this construction, the parental plasmid cannot express the large T antigen, and, therefore, the plasmid DNA cannot replicate (45); however, if recombination occurs within the duplicated segment of pBSVD1969, a complete T antigen gene is created and replication can occur. Accordingly, a comparison was made between the specific infectivities of pBSVD1969 DNA and the duplication plasmids that had an intact early region but required recombination to produce viable virus. pBSVD1969 produced about 2% of the number of plaques that wild-type SV40 DNA produced (Table 1). Moreover, DNA from a representative set of these plaques had the same size and restriction patterns as wild-type structure. Judging from the recombination frequencies found with the other pBSVD plasmids, the value of 2% is only about half of what might be expected for a molecule with about 2,000 bp of homology. This result indicates that neither large T antigen nor DNA replication is essential for intramolecular homologous recombination to occur.

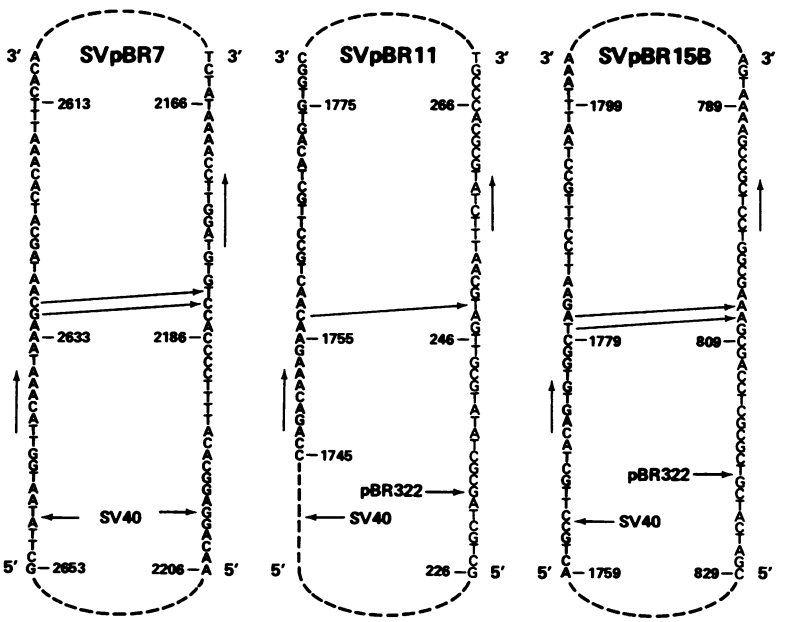

FIG. 6. DNA sequence flanking the recombination junctions in SVpBR7, SVpBR11,and SVpBR15B. The DNA sequence in the 5′ → 3′ direction is shown from bottom to top along the left and switches at the point of recombination (indicated by arrows) to the sequence on the right. A single crossover arrow occurs where a unique recombination event could be assigned (e.g., SVpBR11), and two crossover arrows indicate alternative, nonhomologous recombination events that would generate the same product (e.g., SVpBR7). Recombination to generate SVpBR7 occurred between SV40 sequences of pBSV1A, and the recombinations to produce SVpBR11 and SVpBR15B occurred between SV40 and pBR322 sequences.

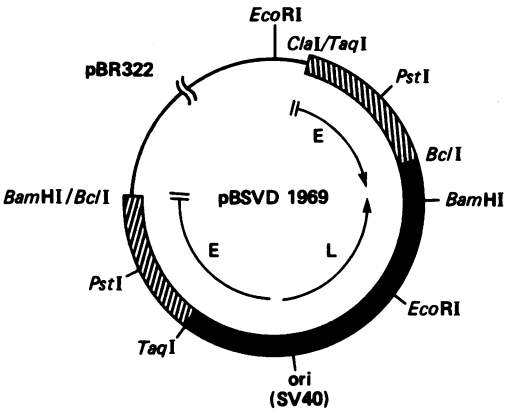

FIG. 7. DNA substrate to test the requirement for replication and large T antigen in homologous recombination. pBSVD1969 has a complete late region, but the early region is interrupted by pBR322 DNA. In addition, the hybrid plasmid contains a duplication (1,969 bp) of a region of SV40 DNA indicated by the hatched segment. Therefore, pBSVD1969 DNA cannot express the T antigen gene or replicate; however, homologous recombination within the duplication would yield a viral genome with a functional early region.

Another type of hybrid plasmid was used to determine whether T antigen and DNA replication are needed for nonhomologous recombination. The substrate for this experiment (pNHR1) contained an insert of about 4.5 kilobase pairs of pBR322 DNA within the large T antigen intron (at the *TaqI* restriction site); the insert also included a segment of SV40 DNA that contained the early region polyadenylation signal (Fig. 8A). The relevant feature of this DNA molecule is that the interruption of the SV40 early region sequence by pBR322 DNA containing a polyadenylation processing signal eliminates, or markedly reduces, the likelihood of producing a functional large T antigen and, therefore, of plasmid DNA replication. However, plaque-forming virus could be produced if the polyadenylation signal and sufficient DNA to yield a nearly wild-type-sized SV40 DNA were eliminated by nonhomologous recombination. Provided that the large T antigen coding sequence is not impaired by the recombination event, the viral genome should be able to multiply and produce plaques. This experimental design assumes that recombinations yielding large T antigen introns of different size and nucleotide com-

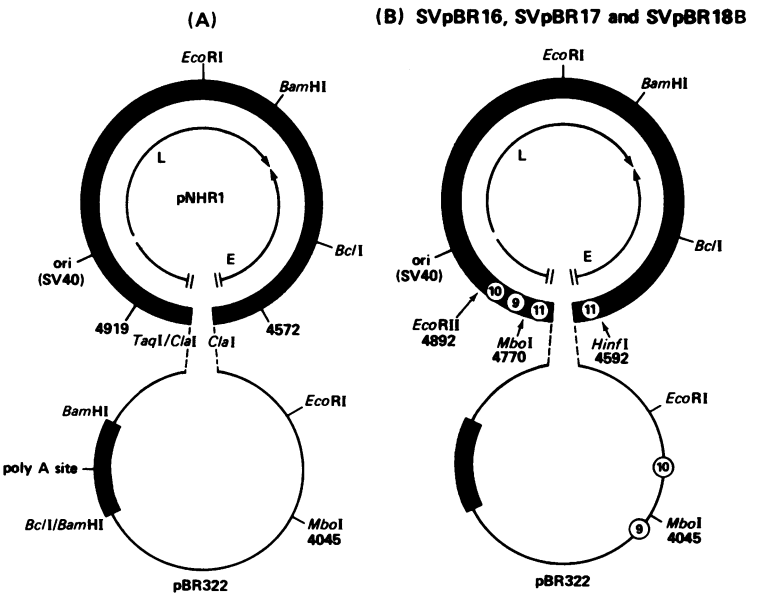

FIG. 8. Substrate to test for the requirement of replication and large T antigen in nonhomologous recombination. pBRSV11 contains a 237-bp *Bam*HI-to-*Bcl*I fragment derived from SV40 DNA inserted at the *Bam*HI restriction site of pBR322. The hybrid plasmid pNHR1 results from the joining of SV40 DNA at its unique *TaqI* restriction site (within the large T antigen intron [12]) to the *ClaI* restriction site of pBRSV11 DNA. The boundaries of the large T antigen intron are indicated by nucleotides 4,919 and 4,572. pNHR1 contains an intact late region, but the early region is interrupted by pBRSV11. (B) Nonhomologous recombination products derived from pNHR1. SVpBR16, SVpBR17, and SVpBR18B were generated by recombination between points labeled 9, 10, and 11, respectively.

position still allow the early region transcripts to be spliced. Consequently, recombinations that remove pBR322 DNA and only dispensible SV40 intron sequences will produce plaque-forming genomes.

Transfections with pNHR1 plasmid DNA yielded plaques in the absence of helper DNA at a frequency of 0.13% relative to wild-type SV40 DNA (Table 1). As emphasized above, this experiment can provide only a minimal estimate of the frequency of recombination, since recombinations that remove large T antigen coding sequence or nucleotides essential for splicing, or insufficient DNA to permit encapsidation of the progeny DNA, would not produce plaque-forming virus genomes. Note that the frequency of plaques is increased nearly 20-fold by coinfection with *tsB*201. This implies that many recombinants with defective early region function are also produced. Although not compelling, the results of this experiment suggest that intramolecular nonhomologous recombination can occur in CV1P cells in the absence of large T antigen and DNA replication.

Structure of recombinant genomes produced from pNHR1 plasmid. Viral DNAs from three independent plaques produced after transfections of CV1P with pNHR1 DNA were examined by restriction enzyme analysis as described earlier. Figure 8B shows the inferred recombination sites: SVpBR16, SVpBR17, and SVpBR18B appear to be joined at sites labeled 9, 10, and 11, respectively. Thus, SVpBR16 and SVpBR17 DNAs have substitutions of pBR322 segments for SV40 sequences within the large T antigen intron; SVpBR18B lacks pBR322 DNA sequences and, therefore, has deleted the entire plasmid from the intron. The boundaries of the SV40 large T intron are at nucleotides 4,919 and 4,572. Interestingly, the junctions in all of the recombinants referred to in Fig. 8B occur within those splice boundaries.

DNA sequence at the recombination sites in SVpBR16, SVpBR17, and SVpBR18B. The products derived from pNHR1 were sequenced by using the Maxam and Gilbert procedure (25). SVpBR16, SVpBR17, and SVpBR18B DNAs were sequenced by using fragments that were ^{32}P end labeled at the SV40 *Eco*RII site at nucleotide 4,892, the pBR322 *Eco*RI site, and the SV40 *Mbo*I site at nucleotide 4,770, respectively. The DNA sequence across the recombination junctions of these DNAs is shown in Fig. 9.

Recombination in SVpBR16 occurred between nucleotide 4,028 or 4,027 of pBR322 and 4,838 or 4,839 of SV40. Although there is no homology at the crossover point, a 4-bp homologous sequence (ACAT) occurs in SV40 and pBR322 DNA, 3 to 4 bp from the recombination junction (Fig. 9). Recombination in SVpBR17 occurred between nucleotides 4,860 in SV40 and 4,107 in pBR322. Only the homology of three A residues 8 to 10 bp from the junction is obvious. SVpBR18B was the only recombination that occurred within a region of homology; thus, the recombination could have occurred anywhere within the 5-bp sequence TTGAT (Fig. 9).

Interestingly, the recombination events in SVpBR17 and SVpBR18B generate the sequences CCTGG and GATTC, respectively; these are recognition sites for the restriction endonucleases *Eco*RII and *Hin*fI. These sites were confirmed by the restriction enzyme analysis.

DISCUSSION

The present studies show how specially designed DNA substrates can be used to analyze homologous and nonhomologous recombination in cultured mammalian cells. Homologous recombination was detected and quantitated by the production of wild-type virus after transfection of CV1P cells with pBR322 plasmids containing duplications of various fractional lengths of SV40 DNA (see Fig. 2). Transfections with plasmids containing a single copy of SV40 DNA interrupted by pBR322 DNA in either the early or the late region allowed us to detect and quantitate nonhomologous recombinations (see Fig. 1). The amount of substrate DNA needed for such experiments is small (1 to 10 μg), and a large number of independent recombinants can be isolated and characterized readily.

It is essential in performing such experiments that the plasmid DNA substrates not contain recombinants generated during their propagation in *E. coli*. Even with recombinant-deficient bacteria (HB101 is $RecA^-$), low-frequency recombination within homologous sequences can confuse the results. In our experiments, Southern blots (38) confirmed that SV40 DNA species smaller than the intended hybrid plasmid substrates were absent. Moreover, if any of the pBSVD DNA had undergone recombinational excision during their preparation, the SV40 DNA would have been lost because it cannot be propagated in *E. coli*.

Transfections of CV1P cells with plasmids containing only a single copy of SV40 DNA, joined at sites within either the early or the late region, did not yield virus plaques. By contrast, comparable transfections with plasmids containing an entire viral genome plus a homologous SV40 DNA segment (see Fig. 2) yielded a substantial number of wild-type SV40 progeny. Indeed, the probability that transfected centers will yield wild-type virus is greater as the length of the homologous segment is increased. Considering that the viral genomes obtained from

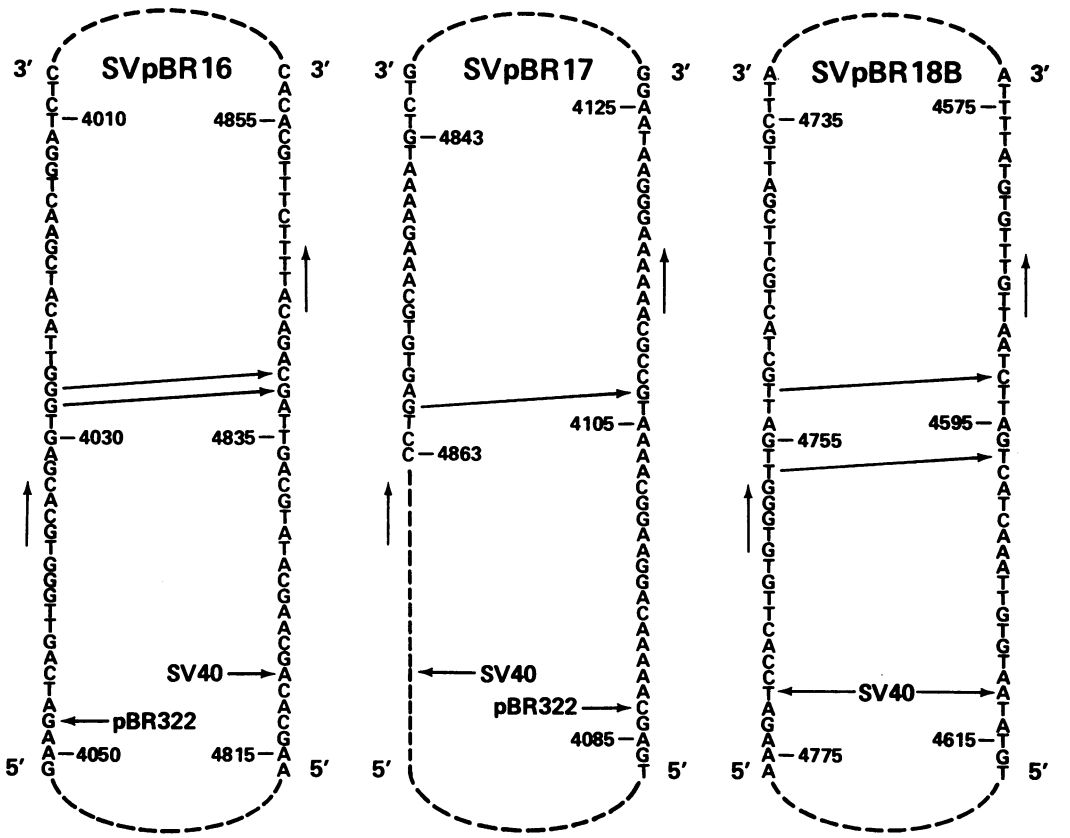

FIG. 9. DNA sequence flanking the recombination junctions in SVpBR16, SVpBR17, and SVpBR18B. Recombination between SV40 and pBR322 sequences generated SVpBR16 and SVpBR17, whereas SVpBR18B can be accounted for by recombination among the SV40 sequences shown. The switch in the DNA sequence from the strand on the left to the strand on the right is indicated by the long arrows. SVpBR18B has a 5-bp homology (TGATT) at the point of recombination. Therefore, recombination could have occurred anywhere in the 5-bp region between the two long arrows.

such transfections are indistinguishable from wild-type virus, they probably arise by recombinations within the homologous segment.

The recombinant progeny obtained from the transfections with the pBSV plasmids indicate that recombination at nonhomologous regions of DNA occurs at an appreciable frequency. The estimate we obtained (2 to 7%) is undoubtedly too low, since nonhomologous recombinations that produce genomes that are too large or too small for encapsidation are not scored; moreover, recombinants with defective early and late regions are not detected as a plaque with either helper. Judging from the frequencies of homologous and nonhomologous recombinations obtained with the same set of plasmids (pBSVD237-5243), the two types of events may be nearly equally probable. With this experimental design, however, it is not possible to obtain a more precise estimate or comparison of either the homologous or the nonhomologous recombination frequency.

The analysis of the nucleotide sequences of the nonhomologous recombinants establishes that the recombination yielding SVpBR7 occurred between nonhomologous sequences in SV40 DNA and that SVpBR8 and SVpBR11 arose by recombination between pBR322 and SV40 sequences. In each case examined so far, the recombination event joined nucleotides present in the substrates without the incorporation of bases whose origin could not be traced. SVpBR7 contains a 448-bp deletion from nucleotide 2,183 to 2,631 in the SV40 late region. This deletion removes the sequence coding for the C-terminal part of VP1, including the termination codon (bp 2,591 to 2,593) and the AAATAA polyadenylation signal for the early mRNAs of SV40 (bp 2,608 to 2,603). Judging by the ability of SVpBR7 to complement *tsA58*, the removal of the polyadenylation signal does not impair the expression of its early RNAs, probably because there is an alternative functional polyadenylation signal (bp 2,638 to 2,633). These results are

consistent with the existence of viable deletion mutants that lack the polyadenylation signal normally used by the SV40 early mRNAs (13). SVpBR11 contains 126 bp of pBR322 sequence and has lost 776 bp from SV40. The entire 126 bp of pBR322 sequence have been confirmed by DNA sequencing (Fig. 5; other data not shown). SVpBR15B contains an insertion of 430 bp from pBR322 and lacks 752 bp from SV40.

In considering the mechanisms of the two types of recombinations, we tested whether large T antigen or replication of the substrate DNA, or both, was necessary. This was done with specifically designed substrates (Fig. 7 and 8) that cannot replicate in monkey cells because large T antigen is not available before a recombination event. pBSVD1969 DNA is unable to induce the formation of the large T antigen and probably the small t protein as well; nevertheless, homologous recombination occurs with only a slightly lower than expected frequency. Therefore, although replication is not essential for homologous recombination, the increased concentration of recombination substrates produced by replication probably accounts for the increased frequency of recombination. This implies that all of the proteins involved in the recombination event are host coded and that replicative structures are not obligatory substrates for the homologous recombination process. The frequency of nonhomologous recombinants obtained with the nonreplicable pNHR1 DNA is about 10- to 20-fold lower than with DNAs that can replicate. One reason to expect a lower frequency with pNHR1 than with the pBSV plasmids is that the target within which the nonhomologous recombination can occur is considerably smaller in pNHR1. Recombinations between pBR322 and SV40 or between SV40 sequences that remove all or most of the pBR322 DNA, but damage the T antigen gene or affect its expression, would not be viable. The fact that some viable virus is produced from pNHR1 indicates that replication of the DNA is not mandatory for it to undergo a nonhomologous recombination event.

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