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Homologous Recombination of Monkey α-Satellite Repeats in an In Vitro Simian Virus 40 Replication System: Possible Association of Recombination with DNA Replication

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To study homologous recombination between repeated sequences in an in vitro simian virus 40 (SV40) replication system, we constructed a series of substrate DNAs that contain two identical fragments of monkey α-satellite repeats. Together with the SV40-pBR322 composite vector encoding Ap^r and Km^r, the DNAs also contain the *Escherichia coli* galactokinase gene (*galK*) positioned between two α-satellite fragments. The α-satellite sequence used consists of multiple units of tandem 172-bp sequences which differ by microheterogeneity. The substrate DNAs were incubated in an in vitro SV40 DNA replication system and used to transform the *E. coli galK* strain DH10B after digestion with *Dpn*I. The number of *E. coli galK* Ap^r Km^r colonies which contain recombinant DNAs were determined, and their structures were analyzed. Products of equal and unequal crossovers between identical 172-bp sequences and between similar but not identical (homeologous) 172-bp sequences, respectively, were detected, although those of the equal crossover were predominant among all of the *galK* mutant recombinants. Similar products were also observed in the in vivo experiments with COS1 cells. The in vitro experiments showed that these recombinations were dependent on the presence of both the SV40 origin of DNA replication and SV40 large T antigen. Most of the recombinant DNAs were generated from newly synthesized *Dpn*I-resistant DNAs. These results suggest that the homologous recombination observed in this SV40 system is associated with DNA replication and is suppressed by mismatches in heteroduplexes formed between similar but not identical sequences.

It is known that the genomic DNA of higher eukaryotes, including mammals, contains large amounts of repetitive DNAs. Among them, α-satellite DNA is known to localize in centromeric regions of primate chromosomes as tandem arrays of thousands of kilobases. Homologous recombinations between these repetitive DNA sequences potentially play a crucial role in drastic chromosomal rearrangements such as chromosomal deletion, duplication, inversion, amplification, and translocation. In this context, it is surprising that such chromosomal rearrangements are usually observed very rarely in mammalian cells. The study of homologous recombination between various repetitive DNA sequences is thought to be one of the keys to understanding how chromosome integrity is maintained during the cell cycle in mammalian cells.

Recent reports have shown that intrachromosomal trinucleotide repeat expansion is the cause of several human genetic diseases: myotonic dystrophy, fragile X syndrome, spinal bulbar muscular atrophy, and Huntington's disease (5, 12–14, 19, 21, 26, 31, 42). These rearrangements are thought to occur by either unequal crossing-over, i.e., crossovers between misaligned repeats, or DNA polymerase slippage, i.e., reassociation of repeats in a misaligned configuration during DNA replication. It is important to understand the mechanism of expansions or diminutions of repeats in mammalian cells.

In *Saccharomyces cerevisiae*, intrachromosomal recombination in the rDNA (genes coding for rRNA) cluster occurs less frequently than expected from the physical length of the region (35). Mitotic recombination between δ sequences also takes place less frequently than expected (20). These observations led us to think that there is a mechanism which suppresses recombination between these repetitive DNA sequences. In fact, mitotic recombination between δ sequences or between rDNA sequences was recently found to be enhanced by a mutation in the *TOP3* gene (46). Recombination between δ sequences or between rDNA sequences is also stimulated by mutations in the *TOP1*, *TOP2*, and *SIR2* genes (6, 15, 18). In *Escherichia coli*, recombination between direct repeat sequences depends on RecA function (9) and is enhanced by a mutation in the *topB* gene (39). Albertini et al. (2) proposed that in *E. coli*, DNA replication plays an essential role in recombination between repeated sequences by the slipped-mispairing mechanism. However, little is known about the mechanism of regulation of homologous recombination between repeated sequences in mammalian cells.

Another kind of recombination is known to take place between similar but not identical DNA sequences (homeologous recombination). A mutation in mismatch repair stimulates ectopic recombination between homologous genes in *S. cerevisiae* (4). There is a barrier to recombination between *E. coli* and *Salmonella typhimurium*, but the barrier is disrupted by mismatch-repair mutations (36). It has been shown that mammalian repetitive DNAs contain small sequence heterogeneities. Monkey α-satellite DNA sequences contain 4 to 9 bp of heterogeneity in every 172-bp unit compared with the consensus sequence (32). When recombination takes place between similar but not identical sequences of a pair of α-satellite units (homeologous recombination), stretches of identical se-

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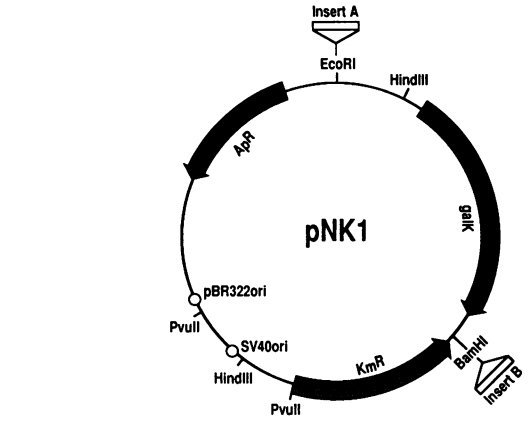
quences vary from very short to relatively long homology (49 bp maximum), depending on the combination of the units. Both homologous and homeologous recombinations can affect the stability of mammalian chromosomes during the course of the cell cycle. Although many analyses to assess the requirement for sequence homology have been done with mammalian systems (28, 45), the nature of homeologous recombination has not yet been well characterized.

We have developed a set of in vivo and in vitro recombination systems using the same substrate DNAs containing α-satellite DNA as target DNAs to analyze the mechanism of homologous and homeologous recombinations in primate cells. The results suggested that intramolecular homologous and homeologous recombinations are associated with DNA replication in vitro. Recombination was frequent in both the in vitro and in vivo systems, suggesting that the recombination observed in the in vitro system is not an artifact and that this in vitro system is well suited for analyzing the mechanism of homologous recombination qualitatively and quantitatively.

MATERIALS AND METHODS

Construction of plasmid pNK1 and its derivatives. We have constructed a shuttle vector plasmid, pNK1, that contains three bacterial markers, *bla* (Ap^r), the galactokinase gene (*galK*), and *neo* (Km^r), as well as the replication origins of pBR322 and simian virus 40 (SV40) DNA (3). To assay homologous recombination, pNK1 derivatives carry a galactokinase gene (*galK*) which is flanked by several kinds of direct or inverted repeats (Fig. 1). Among them, pNK350αDR and pNK960αDR contain two identical 350- or 960-bp fragments, respectively, of α-satellite DNA as a flanking sequence, oriented in the same direction. The α-satellite sequence of 960 bp corresponds to the sequence from bp 81 to bp 912 in Fig. 7 of Ohira et al. (32). pNK1080DR contains two identical 1,080-bp fragments of a unique mammalian genomic DNA sequence (a part of the human DNA topoisomerase II gene) in the same orientation. pNK960αIR contains two identical 960-bp fragments of α-satellite DNA in opposite orientations as a flanking sequence. pNK0-960αDR is identical to pNK960αDR except that the former lacks 340 bp of a *Pvu*II-*Hin*dIII fragment that contains the replication origin of SV40 DNA.

In vitro recombination system. The in vitro recombination system is essentially a modification of the in vitro SV40 DNA replication system developed by Wobbe et al. (48), which contains, in a 50-μl total volume, 0.4 μg of substrate plasmid DNA, affinity-purified SV40 large T antigen (0.5 μg), HeLa cell extract (120 μg of protein; prepared by extraction with 0.2 M NaCl from a HeLa whole-cell homogenate followed by dialysis against low-salt buffer and clarification by high-speed centrifugation), 30 mM Tris-HCl (pH 7.9), 0.5 mM dithiothreitol, 7 mM $MgCl_2$, nucleoside triphosphates (NTPs; 4 mM ATP and 0.2 mM concentrations of each of the others), dNTPs (0.1 mM each, one of which was labeled with ^{32}P at a specific activity of 8 mCi/μmol), and the ATP-regenerating system (20 μg of creatine phosphokinase per ml and 40 mM creatine phosphate). The mixture was incubated for 1 to 6 h at 37°C. After incubation, an aliquot of the reaction mixture was removed to monitor the extent of DNA replication by measuring the incorporation of the ^{32}P-labeled dNTP into the trichloroacetic acid (TCA)-insoluble fraction with a Whatman GF/C filter. The rest of the mixture was treated with 200 μg of proteinase K per ml at 37°C for 30 min in the presence of 20 mM EDTA and 0.2% sodium dodecyl sulfate (SDS). Finally, plasmid DNAs were purified by extraction with phenol-chloroform and repeated ethanol precipitation and dissolved in 10 mM Tris-HCl (pH 7.9)–1 mM EDTA. Purified plasmid DNAs were then used to transform *E. coli* DH10B (*recA galK galU*) before or after digestion with an excess amount of *Dpn*I to assay for rearrangements in the *galK* region of the plasmid DNAs. Ap^r Km^r DH10B transformants were selected on both MacConkey galactose plates and LB plates (both containing 50 μg of ampicillin and 100 μg of kanamycin per ml) to score the number of *galK* Ap^r Km^r colonies and total Ap^r Km^r colonies, respectively. Colonies on MacConkey galactose plates were picked up, and structural alterations of the plasmid DNAs were analyzed as described below. DH10B is *galU galK* as well as *recA*. When DH10B is transformed with intact pNK1 or its derivative DNAs that have functional *galK* genes, it cannot grow on medium containing galactose as a single carbon source, such as a MacConkey galactose plate, because the *galU*



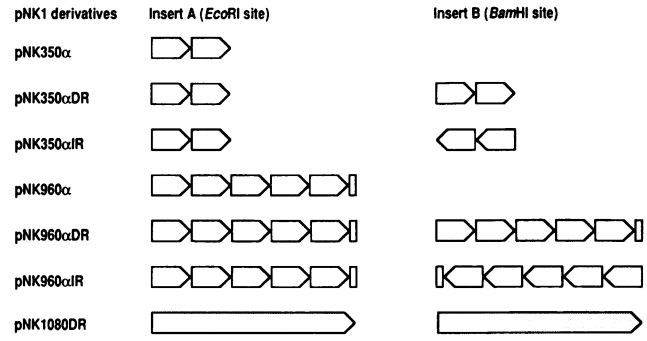


FIG. 1. Structures of substrate DNAs. Shuttle vector plasmid pNK1 contains three bacterial markers, *bla* (Ap^r), *neo* (Km^r), and the galactokinase gene (*galK*), in addition to the replication origins of pBR332 and SV40. On the pNK1 derivatives, the galactokinase gene (*galK*) is flanked by several kinds of direct or inverted repeats. pNK350α and pNK960α contain a 350- or 960-bp fragment, respectively, of α-satellite DNA at the *Eco*RI site. pNK350αDR and pNK960αDR contain two identical 350- or 960-bp fragments, respectively, of α-satellite DNA oriented in the same direction as a flanking sequence at the *Eco*RI and *Bam*HI sites. pNK350αIR and pNK960αIR contain two identical 350- or 960-bp fragments, respectively, of α-satellite DNA oriented in opposite directions as a flanking sequence, at the *Eco*RI and *Bam*HI sites. pNK1080DR contains two identical 1,080-bp fragments of a unique mammalian genomic DNA sequence (a part of the human topoisomerase II gene) as a direct-repeat sequence at the *Eco*RI and *Bam*HI sites. Open shapes represent 172 bp of α-satellite DNA sequence. Shaded shapes represent 1,080 bp of a unique human genomic DNA sequence.

gene is still defective and the transformant cannot metabolize the accumulating toxic metabolite galactose-1-phosphate produced by galactokinase, the product of the *galK* gene on the pNK1 derivative DNAs. Thus, *E. coli* DH10B cells transformed with pNK1 derivative DNAs which are altered to be defective in *galK* can only survive on MacConkey galactose plates containing ampicillin and kanamycin.

Transfection of plasmid DNAs into COS1 cells. Transfection of plasmid DNAs into simian COS1 cells was done by using a modification of the DNA-calcium phosphate coprecipitation method (7). Cells were plated in 100-mm dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and incubated at 37°C. When the cells reached about 70% saturation, calcium phosphate precipitate containing 5 μg of pNK1 or its derivative DNA was added to each plate and allowed to stand for 4 h. Transfected cells were then treated with 20% glycerol in phosphate-buffered saline (PBS) for 1 min, washed with PBS, overlaid with fresh DMEM supplemented with 10% fetal bovine serum, and further incubated at 37°C for 48 to 60 h before the extraction of extrachromosomal DNA.

Recovery of plasmid DNAs from transfected COS1 cells. Plasmid DNAs were extracted by the method of Hirt (16). Low-molecular-weight extrachromosomal DNAs in the Hirt supernatant were treated with proteinase K, extracted with phenol-chloroform, and precipitated twice with ethanol. To remove unreplicated DNAs, the recovered DNAs were incubated with an excess amount of *Dpn*I. DNAs replicated in primate cells should be resistant to *Dpn*I digestion because they lack methyladenine at the recognition sites for this restriction enzyme.

Screening of recombinant plasmid DNAs produced in the in vivo system. Recovered plasmid DNAs were introduced into *E. coli* HB101 (*recA galK*), and transformed bacteria were selected on MacConkey galactose plates containing ampicillin (50 μg/ml) and kanamycin (100 μg/ml). *galK* colonies, which are white, were picked from the red *galK*$^+$ colonies, and the structures of the plasmid DNAs were analyzed as described below.

Structural analysis of *galK* recombinants. *galK* Km^r Ap^r colonies on MacConkey galactose plates were picked, and plasmid DNAs were extracted by a microscale alkali lysis method on 96-well microtiter plates. DNAs were then analyzed by agarose gel electrophoresis after digestion with appropriate restriction endonucleases (mostly doubly digested with *Eco*RI and *Bam*HI) to distinguish deletions produced by homologous or homeologous recombination from point mutations or rearrangements produced by illegitimate recombination. Restriction DNA fragments carrying recombination junctions were cut from the agarose gel, purified, and cloned into both pUC118 and pUC119 cloning vectors for DNA sequencing analysis. DNA sequences were determined by the dideoxynucleotide chain termination method with single-stranded template DNAs.

Other methods. *E. coli* cells were transformed by electroporation by the protocol of Bio-Rad. Southern blotting analysis was performed as specified by the manufacturer (Amersham) with a positively charged nylon membrane (Hybond-N$^+$). Probe DNA was labeled with [α-^{32}P]dATP with the multiprime DNA labeling kit (Amersham).

RESULTS

Detection of homologous recombination in an in vitro SV40 DNA replication system. To develop an in vitro system for mammalian homologous recombination, we tested whether an in vitro SV40 DNA replication system has the ability to mediate homologous recombination. As a substrate for recombination, we constructed pNK960αDR, which contains two identical 960-bp fragments of monkey α-satellite DNA oriented in the same direction. To monitor recombination, the *E. coli* galactokinase gene (*galK*) was inserted between two α-satellite repeats (Fig. 1). pNK960αDR DNA was incubated at 37°C for various periods in the in vitro SV40 DNA replication system developed by Wobbe et al. (48), which contains affinity-purified SV40 large T antigen and HeLa cell extract in addition to $MgCl_2$, NTPs, dNTPs (one of which is labeled with ^{32}P), and an ATP-regenerating system. After the incubation, an aliquot of each sample was removed to monitor the extent of DNA replication either by measuring incorporation of the labeled dNTP into the TCA-insoluble fraction or by autoradiography of the products electrophoresed on an agarose gel. Analysis of TCA-insoluble radioactivity showed that DNA synthesis of pNK960αDR occurred linearly for 1 h and continued up to 4 h, although its rate declined gradually (Fig. 2). Agarose gel electrophoresis analysis showed that the majority of the replication products were RFI, RFII, and high-molecular-weight DNA (data not shown).

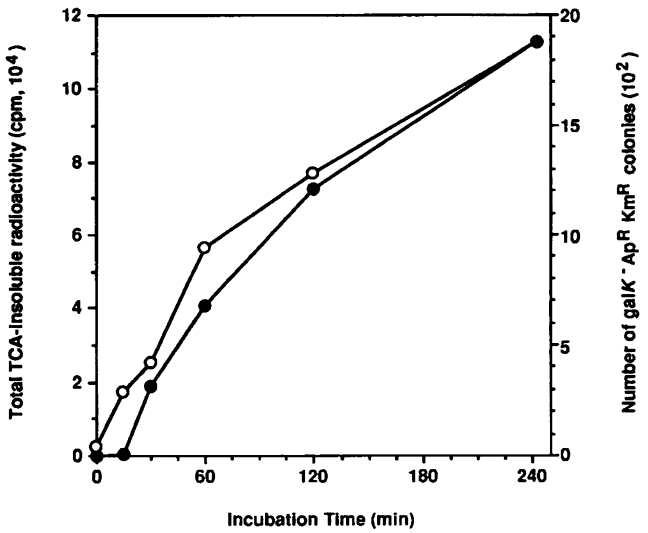


FIG. 2. Time course of DNA synthesis and *galK* mutant plasmid formation in the in vitro system. After pNK960αDR was incubated in the in vitro SV40 DNA replication system for various periods, an aliquot (1/5 volume) of each sample was taken, and incorporation of ^{32}P-labeled dCTP into the TCA-insoluble fraction was measured (open circles). DNA was extracted from the rest of the sample and used to transform *E. coli* DH10B after digestion with an excess amount of *Dpn*I. The number of *galK* Ap^r Km^r colonies was scored on MacConkey galactose plates containing ampicillin and kanamycin (solid circles).

To examine whether homologous recombination took place, DNAs were purified from the reaction mixtures and used to transform *E. coli* DH10B after digestion with an excess amount of *Dpn*I. Ap^r Km^r DH10B transformants were selected on both MacConkey galactose plates and LB plates both containing ampicillin and kanamycin to score the number of *galK* Ap^r Km^r colonies and total Ap^r Km^r colonies, respectively. *E. coli* transformants that carry a plasmid with a functional *galK* gene cannot grow on galactose plate because of the *galU* mutation, which causes accumulation of the toxic product galactose-1-phosphate. Thus, the *galK* mutation was efficiently detected. *galK* Ap^r Km^r colonies were obtained from all DNA samples

TABLE 1. Frequency of *galK* Ap^r Km^r recombinant colonies in the in vitro system

Substrate DNA	SV40 large T antigen present	No. of Ap^r Km^r colonies recovered	No. of *galK* Ap^r Km^r colonies recovered	Recombination frequency (*galK* Ap^r Km^r/total Ap^r Km^r colonies)
Before *Dpn*I digestion				
pNK0-960αDR	+	8.0×10^5	290	3.6×10^{-4}
pNK1	+	6.4×10^5	20	3.1×10^{-5}
pNK350αDR	+	6.5×10^5	115	1.8×10^{-4}
pNK960αDR	+	5.7×10^5	385	6.8×10^{-4}
pNK1080DR	+	4.6×10^5	160	3.5×10^{-4}
After *Dpn*I digestion				
pNK0-960αDR	−	$<1.0 \times 10^2$	<3	ND[a]
	+	5.0×10^2	<5	ND
pNK1	−	4.0×10^2	<3	ND
	+	2.7×10^4	18	6.7×10^{-4}
pNK350αDR	−	7.0×10^2	<3	ND
	+	5.1×10^4	348	6.8×10^{-3}
pNK960αDR	−	1.0×10^2	<2	ND
	+	8.3×10^4	1,395	1.7×10^{-2}
pNK1080DR	−	1.0×10^2	<3	ND
	+	1.2×10^5	568	4.7×10^{-3}

[a] ND, not determined.

incubated for longer than 30 min, and the numbers increased proportionally to the increase in TCA-insoluble radioactivity for up to 4 h (Fig. 2). The frequency of *galK* Ap^r Km^r colonies among total Ap^r Km^r colonies was 1.5×10^{-3} at 4 h, which was 28 times higher than that of unincubated control pNK960αDR DNA (5.2×10^{-5}). These results suggested that the formation of *galK* mutant plasmid DNAs, which were shown below by structural analysis to be produced exclusively by homologous recombination, is associated with the process of SV40 DNA replication in the in vitro system.

It was possible that recombination took place after introduction of the DNAs into *E. coli* cells. To examine this, we tried to detect recombinant DNA directly by physical assays, such as Southern blotting and PCR analysis, but these experiments have not succeeded so far. This possibility is, however, unlikely, because the in vitro and in vivo recombination products had several common features and the recombinant DNAs were detected in the *E. coli recA* mutant, which is defective in the early step of homologous recombination, i.e., homologous pairing of DNA molecules. We therefore concluded that at least the early step of homologous recombination takes place in the in vitro recombination system.

Dependence of the formation of *galK* mutant plasmid DNAs on both SV40 *ori* and large T antigen in the in vitro system. To examine further whether *galK* mutant plasmid DNA formation caused by homologous recombination is associated with its DNA replication, we tested several different substrate DNAs in the in vitro system in the presence and absence of SV40 large T antigen. DNAs were incubated at 37°C for 2 h in the in vitro system, treated with proteinase K, and purified as described in Materials and Methods. The resulting DNAs were assayed for the formation of *galK* mutant DNAs by transformation of *E. coli* DH10B after digestion with *Dpn*I and then analyzed structurally. As substrate DNAs, we used pNK1 and its derivative DNAs, which contain a galactokinase gene (*galK*) flanked by several kinds of direct or inverted repeats (Fig. 1).

When the substrate DNAs were incubated in the in vitro system without SV40 large T antigen, we could not detect either *galK* Ap^r Km^r colonies or Ap^r Km^r colonies after *Dpn*I digestion, suggesting that the substrate DNAs tested neither replicated nor recombined in the absence of SV40 large T antigen (Table 1). When the substrate DNAs were incubated in the presence of SV40 large T antigen, we could detect *galK* Ap^r Km^r colonies at high frequencies with each substrate except pNK0-960αDR after *Dpn*I digestion. When pNK0-960αDR, which does not contain the replication origin of SV40, was used as a substrate, we could not detect either *galK* Ap^r Km^r colonies or Ap^r Km^r colonies after *Dpn*I digestion even in the presence of SV40 large T antigen. The frequency of *galK* Ap^r Km^r colonies among total Ap^r Km^r colonies after *Dpn*I digestion was low for pNK1 (6.7×10^{-4}), medium for pNK350αDR (6.8×10^{-3}) and pNK1080DR (4.7×10^{-3}), and maximum for pNK960αDR (1.7×10^{-2}) (Table 1). We repeated the above set of experiments twice more and got the same results (data not shown). These results indicate that the homologous recombination which is detected by the *galK* mutation assay is dependent on both the large T antigen and the replication origin of SV40 and suggest that homologous recombination is closely associated with SV40 DNA replication in this in vitro system.

It should be noted that when pNK1 derivative DNAs containing the replication origin of SV40 were incubated in the system containing SV40 large T antigen, the number of *galK* Ap^r Km^r colonies did not change or even increased after *Dpn*I treatment, whereas the number of total Ap^r Km^r colonies decreased after *Dpn*I treatment to 4 to 26% of the number obtained without *Dpn*I treatment. These results suggest that only a minority of the substrate DNAs were replicated in this in vitro system and that most of the *galK* mutant DNAs were generated from these replicated minority DNAs during the course of or after their DNA replication (Table 1).

Analysis of structures of *galK* mutant plasmid DNAs formed in the in vitro system. To verify that the formation of *galK* mutant plasmid DNAs in this in vitro system is mediated by homologous recombination, the structures of the *galK* mutant plasmid DNAs were analyzed. Among the *galK* plasmid DNAs, those generated from pNK960αDR, which contains direct repeats of a 960-bp α-satellite DNA sequence consisting of 5.3 units of tandem 172-bp sequences, were analyzed intensively. After *E. coli* DH10B was transformed by pNK960αDR incubated in the in vitro system, *galK* Ap^r Km^r colonies on MacConkey galactose plates with ampicillin and kanamycin plates were picked, and plasmid DNAs were prepared from them by the microscale alkali lysis method. DNAs were then

analyzed by agarose gel electrophoresis after double digestion with *Eco*RI and *Bam*HI. The parent pNK960αDR DNA generates two 0.96-kb fragments of α-satellite DNA sequence, a 2.3-kb fragment containing the *galK* gene, and a 4.0-kb fragment containing the rest of the plasmid. If a deletion of the *galK* gene occurs by homologous recombination between two flanking 0.96-kb α-satellite DNA sequences, the double digestion will generate a 0.96-kb fragment of α-satellite DNA sequence containing a recombination junction and a 4.0-kb fragment containing pNK1 sequence other than the *galK* gene. This type of recombinant DNA is produced by recombination between identical 172-bp sequences and is referred to as the product of an equal crossover event. It is also possible that this substrate DNA will be subjected to unequal crossovers, which occur between similar but not identical (homeologous) sequences within two arrays of 0.96-kb α-satellite DNA sequences. In these cases, the double digestion will generate one fragment whose length is determined by an arithmetic progression from 172 bp (272, 444, 616, 788, 1,132, 1,304, 1,476, and 1,648 bp) of α-satellite DNA sequence containing a recombination junction and a 4.0-kb fragment containing pNK1 sequence other than the *galK* gene. These types of recombinant DNAs are referred to as the products of an unequal crossover event. By these analyses, *galK* deletion mutations produced by homologous or homeologous recombination (equal crossover or unequal crossover) were distinguished from point mutations or rearrangements produced by illegitimate recombination.

Among 93 *galK* mutant plasmid DNAs thus analyzed, 44, 42, 5, and 2 were classified as products of equal crossover, unequal crossover, point mutation, and illegitimate recombination, respectively. The numbers of the *galK* recombinant DNAs carrying a DNA fragment of α-satellite DNA sequence of 272, 444, 616, 788, 960, 1,132, 1,304, 1,476, and 1,648 bp were plotted against their lengths (Fig. 3a). The number of *galK* recombinant DNAs carrying a DNA fragment of five units (960 bp) of α-satellite DNA was sixfold higher than that of recombinant DNAs carrying one of four (788 bp) or six (1,132 bp) units of α-satellite DNA. It was therefore indicated that homologous recombination (equal crossover) occurred significantly more often than any of the homeologous recombinations (unequal crossover).

To demonstrate that the sizes of DNA fragments of the α-satellite DNA sequence in the *galK* mutant recombinant DNAs are discrete, the double-digestion pattern of three independently isolated DNA products of each class of equal and unequal crossovers on agarose gel electrophoresis was determined (Fig. 4a). It can be seen that three independent DNA products of each class are all identical in size, and all of them constitute an arithmetic progression of 172. To confirm the origin of DNA fragments constituting the arithmetic progression, DNAs were transferred to a positively charged nylon membrane and hybridized to a ^{32}P-labeled α-satellite DNA probe, and it was ascertained that all of these fragments are derived from the α-satellite DNA sequence (Fig. 4b). Finally, to make sure that these DNA fragments were really produced by equal and unequal crossovers, representative restriction DNA fragments of each class (272, 444, 616, 788, and 960 bp), which should carry recombination junctions, were cloned into both pUC118 and pUC119 cloning vectors for DNA sequencing analysis. From the sequences determined, all of the cloned DNA fragments were confirmed to have recombination junctions generated by either equal or unequal crossover, as expected. Furthermore, based on the sequences of the recombination junctions of unequal crossovers, we could confine the regions of crossing-over between homeologous α-satellite DNA sequences by using their small heterogeneities (Fig. 5A and 6A). We found that in one of the two arrays of 960-bp α-satellite DNA sequence on pNK960αDR (one of which is located at the *Bam*HI site), a specific 172-bp unit (the second unit in the array) was predominantly used as a crossing-over region in three of four junctions sequenced, but we could not find a specific subregion used predominantly as a crossing-over site within the 172-bp sequence (Fig. 5A and 6A). From all of these structural analyses, it was confirmed that most of the *galK* mutant plasmid DNAs derived from pNK960αDR were certainly produced by either equal or unequal crossover events (86 of 93).

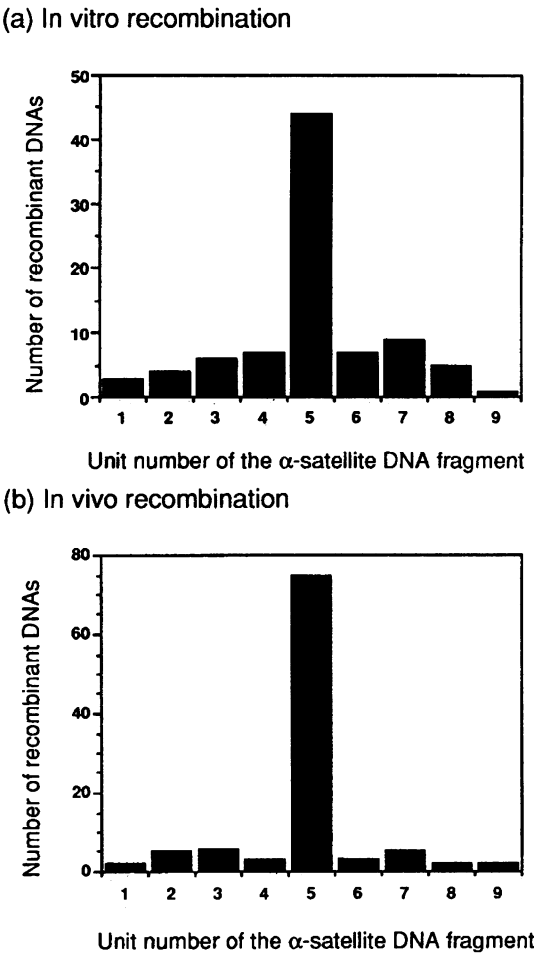


FIG. 3. Distribution of equal and unequal crossovers. (a) *galK* mutant recombinant DNAs found in the in vitro system. (b) *galK* mutant recombinant DNAs found in the in vivo system. The numbers of the *galK* recombinant DNAs carrying a DNA fragment of α-satellite DNA sequence of 272, 444, 616, 788, 960, 1,132, 1,304, 1,476, and 1,648 bp were plotted against their lengths, which were represented as the number (n) of α-satellite DNA units (172 bp). The length (l) is given as l (base pairs) = $172n + 100$ ($1 \leq n \leq 9$).

Analysis of in vivo plasmid recombination in COS1 cells. To examine whether the mode of recombination in the in vitro system reflects that of in vivo plasmid recombination, we studied the effect of direct repeats on the frequency of deletion formation in the *galK* gene of pNK plasmid DNAs transfected into monkey COS1 cells. pNK plasmid DNAs were introduced into COS1 cells by the DNA-calcium phosphate coprecipitation method and then incubated for 48 to 60 h at 37°C. During these periods, pNK plasmid DNAs which contain the replica-

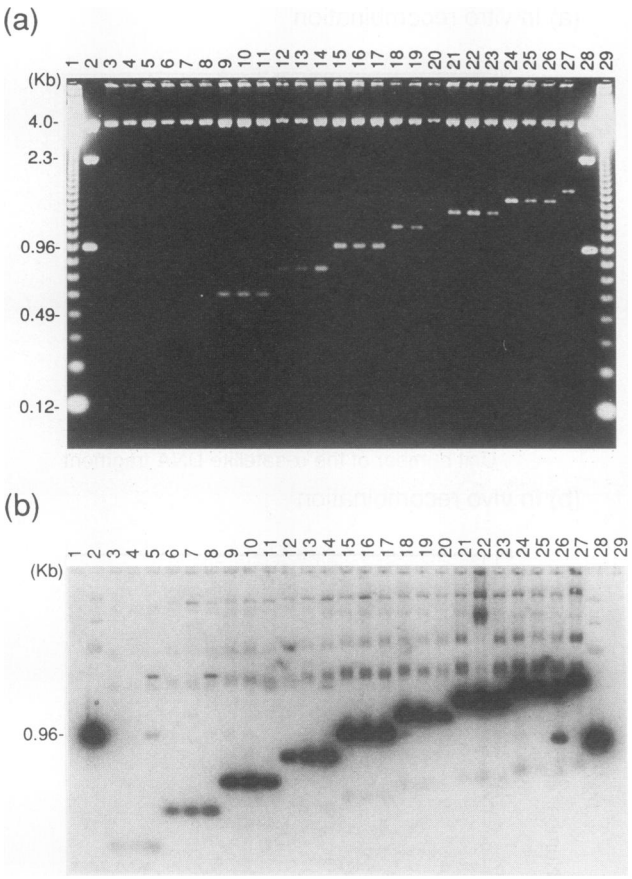


FIG. 4. Electrophoretic analysis of the structures of *galK* recombinant DNAs produced in the in vitro system. Three representative DNA products from each class of equal and unequal crossovers were digested by both *Eco*RI and *Bam*HI and subjected to 1.6% agarose gel electrophoresis. (a) Ethidium bromide staining. Lane 1, 123-bp ladder as size markers; lane 2, pNK960αDR; lanes 3 to 5, recombinants containing one unit of α-satellite DNA (272 bp); lanes 6 to 8, recombinants containing two units of α-satellite DNA (444 bp); lanes 9 to 11, recombinants containing three units of α-satellite DNA (616 bp); lanes 12 to 14, recombinants containing four units of α-satellite DNA (788 bp); lanes 15 to 17, recombinants containing five units of α-satellite DNA (960 bp); lanes 18 to 20, recombinants containing six units of α-satellite DNA (1,132 bp); lanes 21 to 23, recombinants containing seven units of α-satellite DNA (1,304 bp); lanes 24 to 26, recombinants containing eight units of α-satellite DNA (1,476 bp); lane 27, a recombinant containing nine units of α-satellite DNA (1,648 bp); lane 28, pNK960αDR; lane 29, 123-bp ladder as size markers. (b) Southern blotting analysis. DNAs on the agarose gel shown in panel a were transferred to a nylon membrane and hybridized to a ^{32}P-labeled α-satellite DNA probe. DNA samples in lanes 1 to 29 are the same as in panel a.

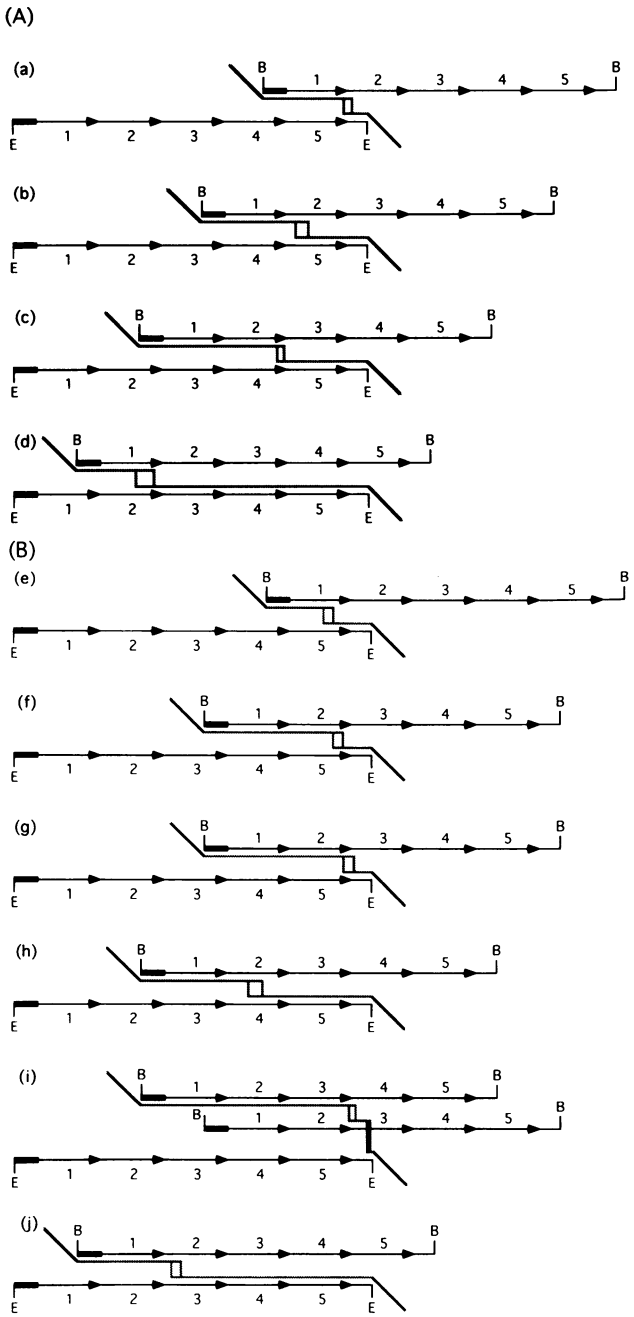


FIG. 5. Schematic representations of unequal crossovers. (A) Unequal crossovers found in the in vitro system. (B) Unequal crossovers found in the in vivo system. Numbered arrows represent α-satellite DNA units which differ by microheterogeneity. Solid boxes represent a part (48 bp) of the *E. coli galK* gene contained in the 960-bp α-satellite DNA fragment. B, *Bam*HI; E, *Eco*RI. Stippled lines represent structures of unequal crossover products; horizontal lines represent α-satellite DNA sequences in the products; oblique lines represent the rest of the structures; pairs of vertical lines represent boundaries of confined regions of crossing-over. Unequal crossover products a, b, c, d, e, f, g, h, i, and j contain 1, 2, 3, 4, 1, 2, 2, 3, 3, and 4 units of α-satellite DNA sequence, respectively. Product i was generated by two consecutive unequal crossovers.

tion origin of SV40 were allowed to carry out multiple rounds of DNA replication, depending on the functions of SV40 large T antigen and host replication enzymes. pNK plasmid DNAs were then extracted by the method of Hirt (16) and used to transform *E. coli* HB101 (*galK recA*). The frequencies of deletion formation in the *galK* gene of the pNK plasmid DNAs could be scored as the ratio of white *galK* Ap^r Km^r colonies to total Ap^r Km^r colonies on MacConkey galactose plates containing ampicillin and kanamycin. With this assay system, it was shown that the frequency of *galK* deletion was increased in plasmids carrying direct repeats but not inverted repeats, and

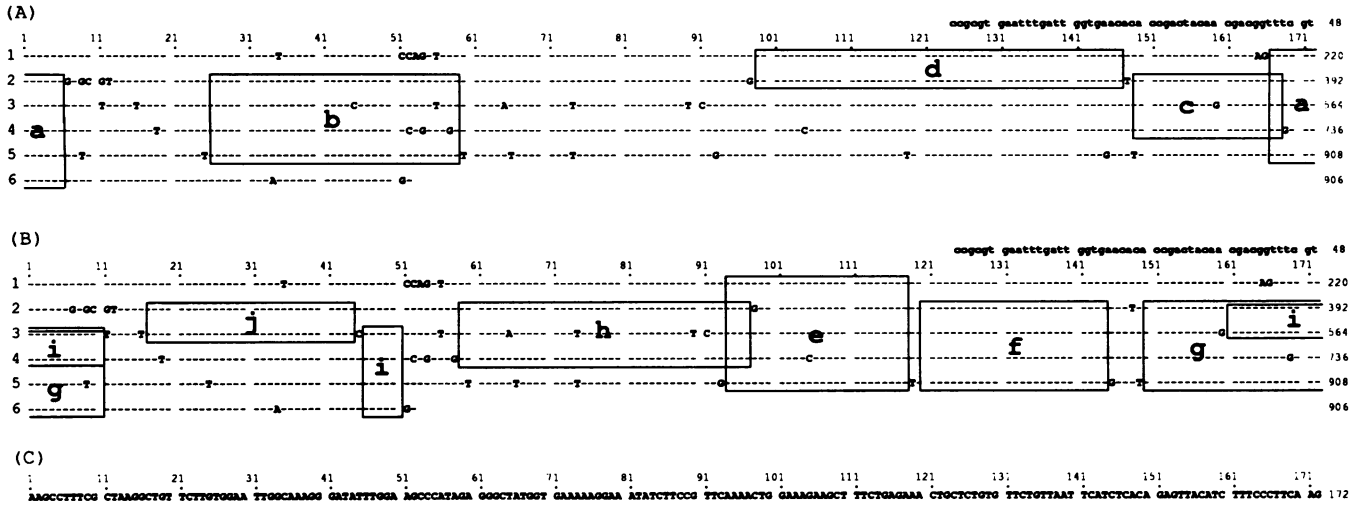


FIG. 6. Sequences of crossing-over regions of *galK* mutant recombinant DNAs produced by unequal crossover. (A) Sequences of crossing-over regions of *galK* recombinant DNAs found in the in vitro system. (B) Sequences of crossing-over regions of *galK* recombinant DNAs found in the in vivo system. Boxed sequences indicate confined regions of crossing-over deduced from comparisons of sequences between galK recombinant DNAs and the original 960-bp α-satellite DNA. The highest and lowest sequences in the boxes represent the regions in which crossovers took place. Boxes labeled a to j correspond to the unequal crossovers a to j shown in Fig. 5. The five lines numbered on the left (1 to 5) indicate 172-bp α-satellite DNA unit sequences and correspond to the numbered arrows in Fig. 5. The bottom line indicates 52 bp of a truncated sixth α-satellite DNA unit sequence. Uppercase letters, bases different from the consensus sequence; bars, bases identical to the consensus sequence; lowercase letters on the top line, 48 bp of *galK* sequence. Numbers on the right indicate the coordinates of the 960-bp α-satellite DNA sequence. (C) Consensus sequence of the 172-bp α-satellite DNA unit.

the frequency was dependent upon the length of the direct repeats (Table 2). When 350-bp and 960-bp fragments of α-satellite DNA derived from monkey COS1 cells (32) were used as direct repeats (pNK350αDR and pNK960αDR, respectively), the frequency of *galK* deletion reached 1.7 and 4.8%, respectively, which was more than 2.5 and 7.1 times higher, respectively, than that of pNK1 containing no direct repeat. The effect of direct repeats on *galK* deletion was also seen when a unique mammalian genomic DNA sequence was used as a direct repeat (data not shown).

As in the in vitro study, the structures of the *galK* plasmid DNAs generated from pNK960αDR were analyzed intensively. By the structural analyses, 75, 28, 4, and 28 of the 135 *galK* plasmid DNAs derived from pNK960αDR were classified as products of equal crossover, unequal crossover, point mutation, and illegitimate recombination, respectively. This result indicates that, in the in vivo system, most of the *galK* plasmid DNAs derived from pNK960αDR were also produced by either equal or unequal crossover (103 of 135), as was the case for the in vitro system. The numbers of *galK* recombinant DNAs carrying an α-satellite DNA fragment of 272, 444, 616, 788, 960, 1,132, 1,304, 1,476, and 1,648 bp were plotted against their lengths (Fig. 3b). The number of *galK* recombinant DNAs carrying a DNA fragment of five α-satellite units (960 bp) was approximately 25-fold higher than that of recombinant DNAs carrying one of four units (788 bp) or six units (1,132 bp) of α-satellite DNA. It was therefore indicated that, in the in vivo system, homologous recombination (equal crossover) took place at a much higher frequency than any of the homeologous recombinations (unequal crossovers), as was the case for the in vitro system, although the difference was much larger in the in vivo system.

We have further confirmed by DNA sequencing analysis that all of the analyzed α-satellite DNA fragments of the lengths of the arithmetic progression of 172 derived from in vivo *galK* recombinant DNAs contain recombination junctions generated by either equal or unequal crossover. From the sequences of the recombination junctions of unequal crossovers, we could confine the regions of crossing-over between homeologous α-satellite DNA sequences by using their small heterogeneities (Fig. 5b and 6b), as was done in the in vitro study. Interestingly, we found the same preference for a region of crossing-over as in the in vitro recombination, that is, in one of the two arrays of 960-bp α-satellite DNA on pNK960αDR (the one which is

TABLE 2. Frequency of *galK* Ap^r Km^r recombinant colonies in the in vivo system

Transfected DNA	Total no. of Ap^r Km^r colonies recovered	No. of *galK* Ap^r Km^r colonies recovered	Recombination frequency (*galK* Ap^r Km^r/total Ap^r Km^r colonies)	Relative frequency
pNK1	5,347	36	6.7×10^{-3}	1
pNK350α	5,550	44	7.9×10^{-3}	1.2
pNK350αIR	4,542	48	1.1×10^{-2}	1.6
pNK350αDR	4,826	80	1.7×10^{-2}	2.5
pNK960α	4,365	56	1.3×10^{-2}	1.9
pNK960αIR	2,544	32	1.3×10^{-2}	1.9
pNK960αDR	2,902	139	4.8×10^{-2}	7.1

located at the *Bam*HI site), the same specific 172-bp unit (the second unit in the array) as was seen in the in vitro recombination was predominantly used as a crossing-over region (in five of seven junctions sequenced), but again we could not find a specific subregion used predominantly as a crossing-over site within the 172-bp sequence, as in the in vitro recombination. From these analyses of the in vivo recombination, it was shown that the mode of in vitro recombination well reflects that of in vivo recombination, suggesting that the homologous and homeologous recombinations observed in the in vitro system are not artifacts.

DISCUSSION

It was shown that homologous recombination occurred at high frequency in an SV40 in vitro DNA replication system. This recombination was dependent on the presence of both the SV40 origin of DNA replication and SV40 large T antigen. Most of the recombinant DNAs were generated from replicated substrate DNAs during the course of or after their DNA replication, because most of the recombinant DNAs were *Dpn*I resistant. These results suggest that recombination is associated with replication in the in vitro system. The finding that replication and recombination in adenovirus-infected cells are temporally and functionally related (49) is consistent with our results. The possibility that recombination takes place after introduction of the DNAs into *E. coli* cells seems unlikely, because the in vitro recombination products have essentially the same features as those of in vivo recombination and the recombinant DNAs were detected in the *E. coli recA* mutant, which is defective in the early step of homologous recombination, i.e., homologous pairing of DNA molecules. An activity which cleaves a DNA structure equivalent to the Holliday recombination intermediate has also been detected in a HeLa cell extract (47). It is therefore conceivable that these mammalian in vitro systems are capable of mediating early as well as late steps of homologous and homeologous recombination.

As a substrate for recombination, we constructed pNK960αDR, which carries the *E. coli* galactokinase gene (*galK*) flanked by two identical 960-bp fragments of monkey α-satellite DNA oriented in the same direction. Since the 960-bp α-satellite DNA sequence consisted of 5.3 units of tandem 172-bp sequences which differ by microheterogeneity (4 to 9 bp in 172 bp), it was possible that both equal and unequal crossovers would occur between homologous 172-bp sequences and between homeologous 172-bp sequences. Both the in vivo and in vitro experiments showed that equal crossovers occurred much more frequently than unequal crossovers, although the in vivo experiment showed a much higher frequency of equal crossover than the in vitro experiment. These results suggest that base mismatches formed between homeologous sequences suppress recombination, as was suggested by *E. coli–S. typhimurium* systems (8, 36, 41), *S. cerevisiae* systems (4, 29), and mammalian systems (28, 45).

It has been reported that the rate of recombination is proportional to the length of homology. If it is decreased, the rate of recombination is reduced rapidly and reaches a very inefficient state at a base length called the minimal efficient processing segment (MEPS). Some estimates of MEPS are 23 to 27 bp for wild-type *E. coli* (40) and 200 bp for mammalian cells (25, 37). Since the maximum length of homology is obtained in the case of equal crossover, it is possible that the higher efficiency of the equal crossover event is due not to a complete match of base sequences but to the length of the pairing region. This notion is unlikely because it was shown in the in vivo experiment that the number of recombinant DNAs carrying a DNA fragment of five units of α-satellite DNA (the number of equal-crossover products that contain a five-unit length of pairing region) was 10-fold higher than the number of recombinant DNAs carrying a DNA fragment of four or six units of α-satellite DNA (the number of unequal-crossover products with a four-unit length of pairing region). It is therefore inferred that mismatches in heteroduplex DNA formed between homeologous sequences play some influential role in suppressing homeologous recombination. The suppression of homeologous recombination by base mismatch can be one of the mechanisms for maintaining the integrity of mammalian genomic DNA which contains large amounts of repetitive DNA.

It is surprising that recombination is highly frequent in these in vivo and in vitro systems. One might ask whether there are recombinogenic sequences in our plasmid constructs. This question can be tested in our recombination system, because the 960-bp α-satellite DNA sequence consisted of 5.3 units of tandem 172-bp sequences which differ by 4 to 9 bp among the 172 bp. Sequence analyses of the products of unequal crossover showed that there is no recombinogenic region in the α-satellite DNA sequence. It is known that hypervariable minisatellite DNA is a hot spot for homologous recombination in mammalian cells (43). The Z-DNA sequence motif $d(TG)_{30}$ is also known to stimulate homologous recombination (44). It was recently reported that most of the tumors developing in hereditary nonpolyposis colorectal cancer patients display frequent alterations in $(CA)_n$ and other simple repeated sequences (RER^+ phenotype) (1, 17) and that somatic as well as germ line mutations of a gene, the *mutS* homolog, were identified in RER^+ tumor cells. This *mutS* homolog is therefore likely to cause an instability in dinucleotide repeats and to be responsible for this cancer (10, 22, 34). Our in vitro recombination system will provide a good tool for analyzing the functions of the hot spot sequences as well as the *mutS* homolog.

How is DNA replication associated with recombination? During the course of replication, DNA partly unwinds and serves as a template for leading- and lagging-strand DNA synthesis. This unwound region might be used as a substrate for slipped mispairing of homologous or homeologous sequences, leading to the formation of the products of equal and unequal crossovers, as proposed by Albertini et al. (2). Alternatively, the unwound region might be an appropriate substrate for degradation by an endonuclease to generate protruding single-stranded DNA ends which have complementary sequences, leading to the formation of annealed recombination intermediates of equal or unequal crossover, as was described in the single-stranded DNA annealing model (23). This model has been further supported by a DNA transformation experiment in mammalian cells (24), by a DNA injection experiment in *Xenopus* oocytes (27), and by recombination experiments in *S. cerevisiae* (8, 30, 33, 38). In either case, the recombination observed in the in vivo and in vitro systems must have been enhanced by the mechanism which is linked with DNA replication. It may be important in mammalian cells that some mechanisms suppress chromosomal rearrangements which can be enhanced by DNA replication during the cell cycle.

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