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ILLEGITIMATE RECOMBINATION MEDIATED BY MAMMALIAN DNA TOPOISOMERASE II

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ABSTRACT

To understand the mechanism of illegitimate recombination in mammalian cells, we have examined the recombination role of DNA topoisomerase II (Topo II). We found that purified calf thymus Topo II mediates recombination between two phage λ DNA molecules in an in vitro system. The enzyme mainly produced a linear monomer recombinant DNA that can be packaged in vitro. Novobiocin and anti-calf thymus Topo II antibody inhibit this ATP-dependent recombination. The recombinant molecules contain duplications or deletion, and most crossovers take place between nonhomologous sequences of λ DNA, as judged by the sequences of recombination junctions. In order to study the effects of Topo II on illegitimate recombination in mammalian cells, we have developed a new shuttle vector, pNK1, which contains three bacterial genes, amp(ApR), galK and neo(Km^R). Using this system, we have shown that a Topo II inhibitor, VM26, stimulated deletion formation in pNK1 DNA in monkey COS1 cells. Both in vitro and in vivo results suggest that TopoII participates in illegitimate recombination in mammalian cells.

INTRODUCTION

Rearrangements of chromosomes take place in the genomes of bacteriophages, bacteria, and higher organisms. Deletion, duplication, and many other rearrangements appear to be produced by illegitimate recombination between nonhomologous sequences present on separate DNA molecules or located in various regions of a single DNA molecule (Franklin, 1971; Weisberg and Adhya, 1977). Illegitimate recombination in higher eukaryotes is particularly interesting, because these recombinations are frequently observed in the cells. It is known that many illegitimate recombination events in mitotic cells play a role in

neoplasia (Yunis, 1983; Bishop, 1987). Papova viruses are integrated into or excised from the chromosome by illegimate recombination (Stringer, 1982; Bullock et al., 1984). Exogenously introduced DNA that is replicated autonomously or integrated into host chromosome often carries deletion or duplication in the target DNA (Calos et al., 1983; Razzaque et al., 1983; Roth and Wilson, 1988) Gene amplification might also be initiated by an illegitimate recombination coupled to unscheduled DNA replication. Analyses of illegitimate recombination in higher eukaryotes will provide a clue to understanding the molecular mechanism of these arrangements and perhaps provide a way to develop a method for the targeted integration of genes.

It has been reported that E.coli DNA gyrase and bacteriophage T4 DNA topoisomerase participate in illegitimate recombination in in vitro system (Ikeda et al.,1981; Ikeda,1986). Both enzymes belong to the class of Topo II that participate in topological changes in the state of circular DNA (Gellert et al.,1976; Mizuuchi et al.,1980; Kreuzer and Cozzarelli,1980; Liu et al.,1980). The topoisomerases are also known to bind to double-stranded DNA by generating 4-base pair(bp) staggered breaks (Morrison and Cozzarelli,1979; Gellert et al.,1981). These properties are probably relevant to the mechanism of illegitimate recombination.

Eukaryotic TopoII have been isolated from yeast, *Drosophila, Xenopus,* and mammalian cells (Goto and Wang,1982; Miller et al.,1981; Sander and Hsieh,1983; Shelton et al.,1983; Halligan et al.,1985). These eukaryotic topoisomerases resemble the T4 DNA topoisomerase in many respects including their inability to introduce negative supercoils into circular duplex DNA. In the present communication, we report that purified calf thymus TopoII directly participates in the recombination between two λ DNA molecules in vitro and that TopoII participates in deletion formation in monkey cells.

EXPERIMENTAL PROCEDURES

Bacteria, Bacteriophages, and DNAs.

The bacterial strains used were all derivatives of E. coli K12: Ymel supF was used for the preparation and the assay of total plaque-forming units (pfu). N100 Su⁻recA1 was used for the assay of am⁺ plaque-forming phage. BHB2688 and BHB2690 (Maniatis et al., 1982) were used for the preparation of packaging extracts. Phage λ imm434cI Dam15

Flam96bB538 red3 and λ imm434cI Sam7 Ram5 int6 red3 (Kobayashi and Ikeda, 1977) were used as parental phages in calf thymus Topo II-mediated recombination. They are called λb538DF and λSR, respectively. λ DNA was prepared as described (Kobayashi and Ikeda, 1977). P4 knotted DNA was prepared as described (Liu et al., 1981) with some modification. Plasmid DNA was prepared as described (Maniatis et al., 1982).

Enzyme.

Calf thymus Topo II was prepared as described (Halligan et al., 1985).

In vitro Recombination System.

In vitro recombination was carried out in a mixture (10μl) containing calf thymus Topo II (25 μg/ml), two types of λ DNA (each type at 50 μg/ml), 30 mM Tris·HCl (pH 7.4), 1 mM ATP, 7 mM $MgCl_2$, 10 mM KCl, and 7 mM 2-mercaptoethanol. The reaction was terminated at 60 min by adding 10 vol of TE buffer (10 mM Tris·HCl, pH 7.4/0.1 mM EDTA) containing 0.2% $NaDodSO_4$, and DNA was extracted by phenol and dialyzed against TE buffer. The DNA was then packaged in vitro and assayed for am^+ recombinant plaque-forming phage.

Assay for Recombinant Phage.

Samples from crosses were plated on Ymel for total imm434 plaque-forming phage and on N100 for am^+ imm434 recombinants. The frequency of recombination was defined as the fraction of am^+ plaque-forming phage among total plaque-forming phage.

Mammalian Cell Culture.

Simian COS1 cells were grown in Dulbecco·s modified Eagle·s medium (DMEM) with 10% fetal bovine serum.

Plasmid Construction.

Plasmid pSV2neo (Southern and Berg, 1982) was digested with EcoRI and BamHI, and then the EcoRI-BamHI fragment which contains the genes for amp and neo and replication origins of pBR322 and SV40 DNA was isolated. Similarly, pGS3 (Razzaque et al., 1983) was cut with EcoRI and BamHI, and then the fragment containing E. coli galK gene was

isolated. Ligation of the two fragment yielded a plasmid pNK0. pNK0 was then digested with BamHI and AvaI and the resulting 6.2 kb fragment was recyclized by ligation in the presence of BamHI linker. The resulting plasmid is called pNK1 (Fig. 4).

Isolation of COS1 Cell Lines Containing Persistently Replicating Plasmid DNA.

Transfection of plasmid DNAs into simian COS1 cells were carried out by using the DNA-calcium phosphate coprecipitaion method (Chu and Sharp, 1981). Since the neo gene confers resistance to aminoglycoside antibiotic G418 on mammalian cells, it was possible to select cells carrying persistently replicating pNK1 plasmid by continuous cultivation of the cells in media containing G418. At 48 hr after transfection the cells were trypsinized and re-plated at a density of about 1 x 10^5 cells per 100 mm dish in DMEM/10% fetal bovine serum. After an incubation of 15 hr, G418 was added to the medium at final concentration of 300 μg/ml, and surviving colonies were isolated after 20-30 days.

Treatment of Topoisomerases Inhibitors

Transfected cells were treated with 40 μM VM-26, 4μM m-AMSA, or 4μM camptothecin for 1 hr. For the control tube, reagents were replaced by DMSO. The cells were then washed with DMEM 3 times, overlaid with fresh media, and further incubated.

Recovery of the Plasmid DNA from the Transfected Cells.

At 24 hr after the treatment of drugs, the plasmid DNA was extracted by the method of Hirt (Hirt, 1967). The DNA in the supernant was treated with proteinase K. The solution containing low molecular weight DNAs was extracted by phenol-chloroform, and precipitated by ethanol.

Detection of Mutant Plasmids.

The plasmid DNA extracted from the transfected cells was used to transform E. coli HB101 (recA$^-$, galK$^-$). Transformed cells were spread on MacConkey agar containing 2% of galactose and 100 μg/ml of kanamycin. Bacterial cells with plasmids containing wild-type galK give rise to red colonies, whereas bacterial cells with plasmids

containing a galK⁻ mutation give rise to white colonies. The resulting white colonies were picked up and then were streaked onto the plate containing 50 μg/ml of ampicillin for the ampicillin resistance test.

Other methods.

TopoII was assayed by unknotting of phage P4 DNA (Liu et al., 1981). Antiserum to Topo II was generated in rabbits, and an IgG fraction was purified by ammonium sulfate precipitation and DEAE-cellulose column chromatography (Halligan et al., 1985). Sequence analysis was carried out by the dideoxy method (Sanger and Coulson, 1975).

RESULTS

Calf Thymus Topo II Mediates Recombination Between λ Phage DNAs in vitro.

To detect recombination in vitro, we have used two genetically marked λ phage DNAs, λb538DF and λSR DNAs, as substrates for the recombination reaction. Two λ DNAs (each DNA at 50 μg/ml) were incubated with calf thymus DNA Topo II (25 μg/ml) for 60 min at 28°C, and the DNAs were purified by $NaDodSO_4$ and phenol, dialyzed, and then packaged in vitro. am⁺ recombinant phages were detected at a frequency of 3.1×10^{-5} am⁺ recombinants per total pfu, which is significantly higher that of a reaction mixture without topoisomerase (Table 1). The number of total plaque formers was comparable to that of untreated DNA after incubation of λ DNA with calf thymus Topo II,

Table 1. Conditions for formation of recombinants

Exp.	Conditions	Frequency of *am⁺ imm434* recombinants, no. per no. of total *imm434* pfu	Relative frequency of recombinants
1	Complete mixture	3.1×10^{-5}	1
	− Topo II	7.8×10^{-7}	0.03
	− Mg^{2+}	4.8×10^{-7}	0.02
	− ATP	2.8×10^{-6}	0.09
2	Complete mixture	9.2×10^{-5}	1
	+ Novobiocin (750 μg/ml)	1.3×10^{-5}	0.14
	+ Novobiocin (1.5 mg/ml)	5.9×10^{-6}	0.06
3	Complete mixture	1.1×10^{-5}	1
	+ Antibody (30 μg/ml)	3.7×10^{-7}	0.03

After the recombination reaction, 10-μl DNA samples were mixed with TE buffer containing 0.2% $NaDodSO_4$, extracted with phenol, and dialyzed against TE buffer, and an aliquot of the DNA sample was packaged *in vitro*. Topo II, DNA topoisomerase II; antibody, anti-calf thymus topoisomerase II antibody.

indicating that the enzyme did not affect the viability of phage DNA. Mg^{2+} and ATP were required for the reaction (Table 1). A low level of recombination was detected in the absence of ATP. It is consistent with the observation of ATP-independent relaxation activity in a calf thymus DNA Topo II preparation (Halligan et al., 1985).

We confirmed that the active principle that catalyzes the recombination is the DNA topoisomerase II by examining the effects of novobiocin, an inhibitor of Topo II, and of anti-calf thymus Topo II antibody on the recombination reaction. Novobiocin inhibited the recombination up to 14% of the unreacted sample at a concentration of 750 μg/ml, which is consistent with the inhibition of the Topo II activity (50% inhibition with 200 μg/ml) (Miller et al., 1981) (Table 1). Anti-calf thymus Topo II antibody also inhibited this calf thymus topoisomerase-promoted recombination (Table 1), while it did not inhibit the T4 DNA topoisomerase- promoted recombination (data not shown). We concluded, therefore, that calf thymus Topo II mediates the recombination between two phage λ DNAs.

Molecular Nature of Products in the Recombination Reaction.

To examine the molecular nature of the recombination products, DNA incubated with or without calf thymus Topo II was extracted by the $NaDodSO_4$/phenol method and analyzed by neutral sucrose gradient centrifugation (Fig 1). One main peak of bulk packageable DNA was formed at the position corresponding to the ^{32}P-labeled linear monomer

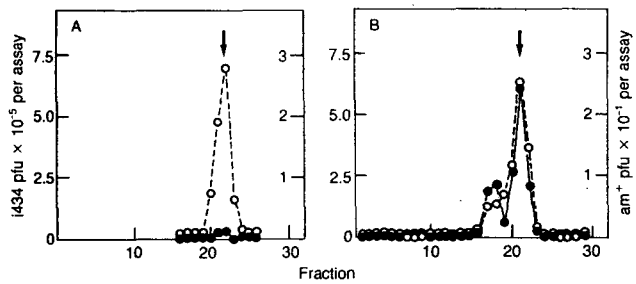

FIG. 1. Neutral sucrose gradient centrifugation analysis of the recombinant DNA. The recombination was carried out as in Table 1. The reaction was terminated by the addition of 0.2% $NaDodSO_4$, and the DNA was treated with phenol and dialyzed. After mixing with unreacted ^{32}P-labeled λ*SR* DNA and heating to 65°C for 10 min, the sample was centrifuged in a 5-ml neutral sucrose gradient [5–10% (wt/vol) sucrose in 50 mM KCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA] at 30,000 rpm for 200 min at 15°C in a Beckman SW50.1 rotor. Each fraction was assayed for *am*$^+$ recombinant DNA activity and for ^{32}P radioactivity. (*A*) Reaction without topoisomerase. The input radioactivity of ^{32}P-labeled DNA was 10^4 cpm, and ≈80% of the labeled DNA was recovered. There were 31 fractions. (*B*) Reaction with 25 μg of calf thymus DNA topoisomerase II per ml. There were 30 fractions. ●, *am*$^+$ *imm434* recombinant pfu; ○, total *imm434* pfu. Arrows indicate the position of ^{32}P-labeled DNA.

DNA in both samples, indicating that the size of the bulk DNA did not change during the Topo II reaction. One main peak of *am*$^+$ recombinant DNA activity was found at the same linear monomer position in topoisomerase, but not in the unreacted sample. This result indicates that the predominant structure of packageable recombinant DNAs formed in the topoisomerase - mediated reaction is a linear monomer and not a nonspecific aggregate or collection of fragments of parental DNA.

Restriction Mapping of the Recombinant Phage DNAs.

To determine the structure of the *am*$^+$ recombinant phage DNAs produced in the *in vitro* recombination and packaging reaction, recombinant DNA was digested by various restriction enzymes and analyzed by agarose gel electrophoresis. As a result of analysis with restriction enzymes, we have mapped the location of the recombination junctions in each of 11 recombinant molecules to within an error of 1.5% (λ map units)(Fig. 2). Fig.2 shows that λ351, λ356, and λ357 have a deletion and the others have a duplication in the λ genome, confirming that most, if not all, of the recombination event promoted by calf thymus Topo II were illegitimate recombination.

Nucleotide Sequences of Recombination Junctions.

We have also determined the nucleotide sequences surrounding the junction in three recombinant phage DNAs. Fig.3 shows the sequences of junctions in the recombinants and the corresponding sequences of parental genomes. In λ351 DNA, a λ sequence occurs up to bp 28,368 and then another λ sequence begins from bp 31,721 (Fig.3a). There is an overlap of 4 bp (GATT/CTAA) at the junction. Hence the crossover could take place at five possible points around these four bases. In λ352 DNA, a λ sequence occurs up to bp 16,924 and then another λ sequence begins from bp 13,818 (Fig. 3b). There is no homology between the parental λ recombination sites. Homology was not detected in the other case in two combinations of the parental recombination sites (Fig. 3c). In summary, among three sets of recombination sites analyzed, there is no homology in two cases and a homology of 4 bp in one case. Therefore, we conclude that homology is not required for the recombination mediated by calf thymus DNA Topo II in *in vitro* reaction.

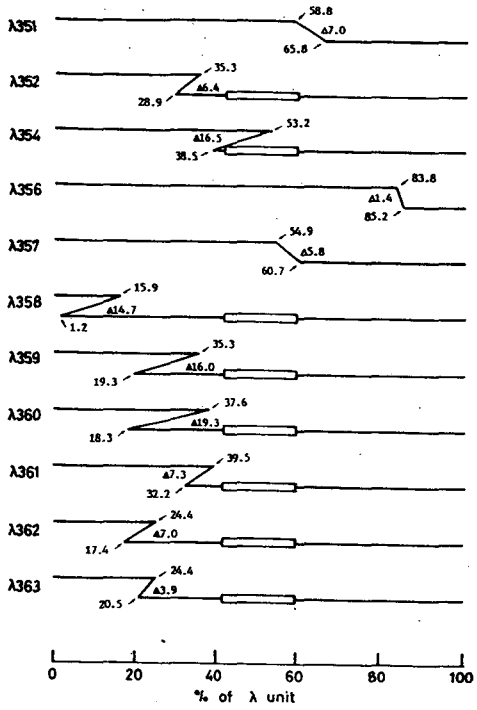

FIG. 2. Structure of the *am*$^+$ recombinant phage DNAs. The recombinant phage DNAs were digested with *Pvu* II, *Sca* I, *Hpa* I, *Ava* I, *Hin*dIII, *Mlu* I, *Acc* I, *Eco*RI, and *Bam*HI. DNAs were electrophoresed in an 0.7% agarose gel, and structures of the recombinants were deduced. Crossover points are shown by arrows with numbers that give the λ map coordinate (left to right, 0 to 100). A solid triangle with its associated number represents the size of the duplication (% of the λ map unit). An open triangle with its associated number represents the size of the deletion (% of the λ map unit). Open rectangle, *b538* deletion originally present on parental λ*b538DF* DNA.

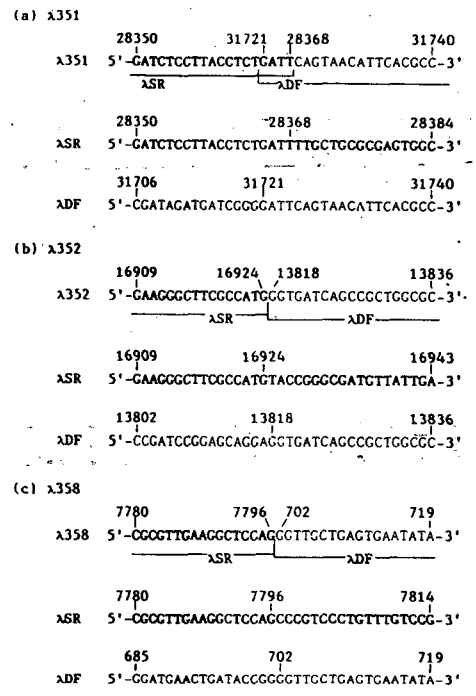

FIG. 3. Nucleotide sequences at the λ–λ junctions. (*a*) Comparison between sequences around the λ–λ junction of λ351 and sequences of the parental λ*SR* and λ*DF* DNAs. (*b*) Sequence of λ352 DNA at the junction and sequences of the parental λ*SR* and λ*DF* DNAs corresponding to the junction. The sites of λ DNA share no common base pair. (c) Sequence of λ358 DNA at the junction and sequences of the parental DNAs. (*d*) Sequence of λ361 DNA at the junction and sequences of the parental DNAs.

Analysis of Deletion Formation in Persistently Replicating Extrachromosomal DNA in Monkey COS1 Cells.

To study deletion formation in mammalian cells, we have constructed a shuttle vector, pNK1, that contains three bacterial markers, amp, galK, and neo and replication origins of pBR322 and SV40 DNA (Fig. 4). Using this vector, deletion occurring during the propagation of pNK1 DNA in monkey COS1 cells can be measured by transfecting the plasmid into E. coli cells and counting the number of galK$^-$ Ap^s double mutants among total Km^R cells.

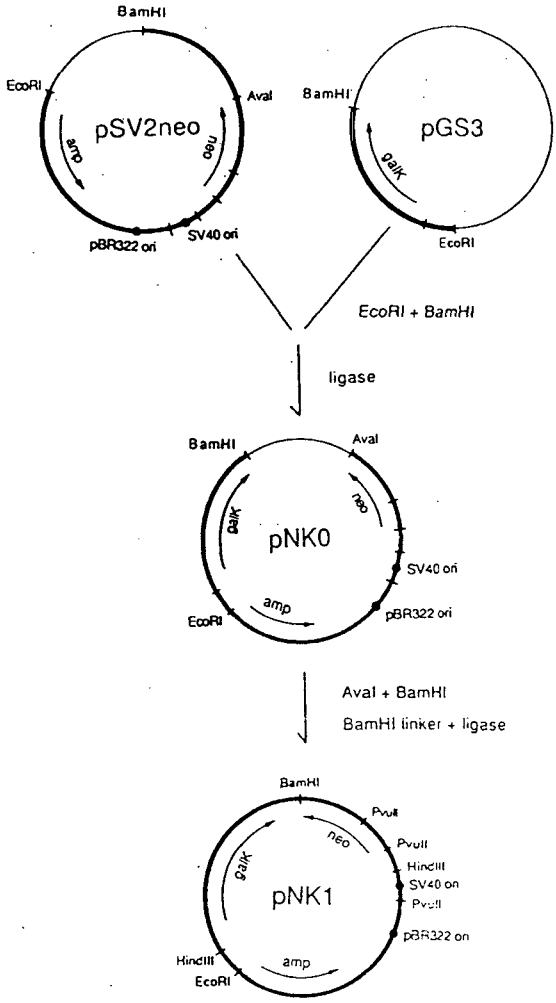

Fig. 4. Construction of pNK1. The *Eco*RI-*Bam*HI fragment of pSV2neo (Southern and Berg, 1982), which contains *bla* (*amp*), *neo* and ons of pBR322 and SV40 DNA, was ligated to the *Eco*RI-*Bam*HI fragment of pGS3 (Razzaque et al., 1983), which contains the *E. coli galK* gene, yielding the plasmid pNK0. pNK0 was then digested with *Bam*HI + *Ava*I and the resulting 6.2-kb fragment was recycled by ligation in the presence of *Bam*HI linkers to give pNK1.

To eliminate the effect of mutations generated during the transfection step, we isolated several lines of stable pNK1 transfectants by continuous cultivation in media containing an aminoglycoside antibiotic, G418. It was found after Southern-blot hybridization that the NEO-11, -13, and -14 cell lines contained extrachromosomally 800, 40, and 20 copies of pNK1-related DNA molecules per cell, respectively (data not shown). Using the COS1 cells containing autonomously replicating plasmid DNA, the frequency of galK$^-$ Ap^s double mutation occurring in pNK1 was examined. The cell lines contained not only the intact pNK1 but also mutant plasmids with mutations in both the galK and the amp genes. In summary, galK$^-$ Ap^s mutant plasmids were detected at frequencis of 17.4%, 8.4%, and 15.5% in the NEO-11, -13, and -14 cells, respectively. Almost all plasmid DNAs (>99%) containing mutations in both the galK and the amp genes were deletion mutants as judged by agarose gel electrophoresis (data not shown). Hence, we regarded the galK$^-$ Ap^s double mutant as a deletion mutant in this

study. Among these cell lines, the NEO-13 cells were used to investigate the effect of topoisomerase inhibitors on deletion formation, because the NEO-13 cells contained a relatively low % of galK⁻ Ap^S mutants.

Effects of DNA Topoisomerase Inhibitors on the Deletion Frequency in pNK1 in COS1 Cells.

To test whether or not the Topo II inhibitors, m-AMSA and VM26 (Nelson et al., 1984; Chen et al., 1984), and the Topo I inhibitor, camptothecin, affect the frequency of deletions in autonomously replicating plasmid DNA during growth of the cells, the NEO-13 cells were treated for 1 h with the drugs. It is expected that DNAs are cleaved by the topoisomerases during treatment with the inhibitors. To allow the cleaved DNA to be rejoined, the drug-containing media were removed and replaced with fresh media. At 24 h after this replacement, the plasmid DNAs were extracted, and then transferred into E. coli HB101 for detection of mutations. The galK Ap^S mutations in the control NEO-13 cells occurred at a frequency of 12% which was higher than the previous value (8.4%), probably because the pNK1 underwent new mutations and/or deletion mutants replicated more rapidly during the two-month cultivation. When the NEO-13 cells were treated with VM26, galK⁻ Ap^S mutants were detected at a frequency of 24.1% per total Km^R colonies (Table 2). Hence VM26 at a concentration of 40 μM increased the frequency of mutation to the untreated cells. In contrast, m-AMSA, the other TopII inhibitor, had no apparent effect on deletion frequency. A Topo I inhibitor, camptothecin, did not affect deletion frequency in our system (Table 2).

Table 2

Effect of topoisomerase inhibitors on the frequency of mutation in the NEO-13 cells

Treatment	Total number of Km^R colonies tested	Number of *galK*⁻ Ap^S colonies	% of *galK*⁻ Ap^S colonies
None	1585	190	12.0 (10.4–13.9)
VM26 (40 μM)	607	146	24.1 (20.2–28.1)
m-AMSA (4 μM)	1570	223	14.2 (12.3–16.3)
Camptothecin (4 μM)	1519	168	11.1 (9.5–12.9)

To determine the structure of the galK⁻ Ap^S mutant plasmid DNAs derived from the VM26-treated cells, the plasmid DNAs were extracted from galK⁻ Ap^S mutant bacteria, digested by various restriction enzymes, and analyzed by agarose gel electrophoresis. As shown in Fig. 5, all of the mutant plasmids contained deletions in the region between the galK and amp genes. We could not find any specificity in the locations of the deletions. This result shows that the deletion mutants were produced by illegitimate recombination.

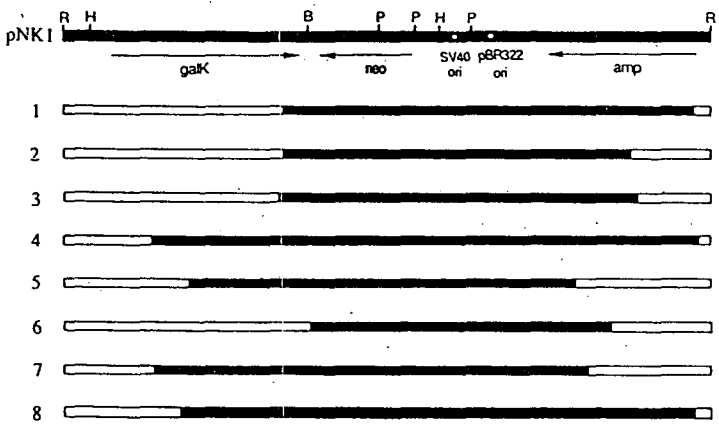

Fig. 5. Restriction mapping of *galK*⁻ Ap^S mutant plasmid DNAs isolated from the VM26-treated NEO-13 cells. The plasmid DNAs were digested with *Bam*HI, *Hae*III, *Hin*dIII, *Hpa*II and *Pvu*II. DNAs were electrophoresed in a 1% agarose or a 5% polyacrylamide gel. Bars 1–8 show the structures of individual deletion mutants isolated from VM26-treated NEO-13 cells. Open bars represent the deleted regions; blackened bars represent the undeleted regions. Total length of pNK1 is 6.2 kb. Restriction sites in pNK1 are shown at the top. B, *Bam*HI; H, *Hin*dIII; P, *Pvu*II; R, *Eco*RI.

DISCUSSION

The present study indicates DNA topoisomerase II has the ability to mediate recombination. Evidence from in vitro reaction supported the interpretation that topoisomerase directly participated in the recombination: Novobiocin and anti-calf thymus Topo II antibody inhibited the reaction. ATP is required for this recombination. Incubation of phage DNAs with the enzyme produced mainly linear monomer recombinant DNA molecules that were packageable in vitro. The

result suggested that calf thymus Topo II is able to complete recombination.

Restriction mapping of recombinant DNA produced in the in vitro reaction indicated that most of the recombinants between two genetically marked phage DNA contained a deletion or a duplication at various sites on the phage DNA, suggesting that the recombination took place between nonhomologous sequences of λ DNA. It is also worthwhile to note that a single crossover can explain the structure of all recombinants. Nucleotide sequences of the crossover sites estimated from the sequences of recombination junctions proved that the crossovers occurred between nonhomologous sites. Therefore, the recombination mediated by calf thymus Topo II did not require homologous DNAs.

Using the monkey COS1 cells containing persistently replicating plasmid DNA, we have shown that a Topo II inhibitor VM26 stimulated deletion formation in mammalian cells. In contrast, m-AMSA had no apparent effect on deletion frequency. The differential effects of these drugs may be due to the difference in the mechanisms of drug action: m-AMSA intercalates into DNA, whereas VM26 interacts with the Topo II (Nelson et al., 1984; Chen et al., 1984). Restriction mapping of deletion mutant plasmid DNA drived from VM26-treated cells indicated that all of the mutant plasmids contained deletion in various regions between the galK and amp genes. This result shows that the deletion mutants were produced by illegitimate recombination.

Mammalian DNA topoisomerase II participates in topological changes in circular DNA, for example, relaxation of supercoiled DNA, formation of catenanes, and knotting or unknotting of circular DNA (Miller et al., 1981; Halligan et al., 1985). The basic mechanism involved in these reaction is a strand-passage reaction that relies on temporary cleavage of double-stranded DNA with a 4-base pair staggered break. The ability to form a double-stranded break might be essential for the recombination.

It has been reported that eukaryotic DNA topoisomerase I can catalyze the ligation in vitro of nonhomologous DNA fragments lacking any sequence homology or complementarity (Been and Champoux, 1983; Halligan et al., 1982). These authors have observed the recombination between single-stranded DNAs or between single-stranded and double-stranded DNAs. Hence Topo II seems to be a more likely

candidate as an enzyme participating in the illegitimate recombination in eukaryotic cells.

Many examples of illegitimate recombination are known in mammalian cells. Simian virus 40 DNA is integrated into or excised from the host chromosome by an illegitimate recombination (Stringer, 1982; Bullock et al., 1984). These integrative or excisive recombinations take place between viral and chromosomal sequences of very short homology. DNA molecules transferred into eukaryotic cells undergo a variety of rearrangements such as deletions and insertions (Razzaque et al., 1983; Calos et al., 1983), or they integrate at various sites on chromosome by an illegimate recombination (Lin et al., 1985; Smith and Berg, 1984). These features resemble those observed in the in vitro recombination mediated by calf thymus DNA Topo II and other topoisomerase. Therefore, it is possible that some, if not all, of the illegitimate recombinations observed in eukaryotic cells may be mediated by the DNA Topo II. The enzyme is distributed generally along the chromosome in Drosophila (Heller et al., 1986) and is a major component of the Drosophila nuclear matrix (Berrios et al., 1985). Therefor, it is conceivable that the topoisomerase II is able to work as an enzyme for illegitimate recombination in eukaryotic cells.

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