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# Bacteriophage T4 DNA topoisomerase mediates illegitimate recombination *in vitro*

**(bacteriophage λ/oxolinic acid/deletion/duplication/subunit exchange model)**

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ABSTRACT **We have found that purified T4 DNA topoisomerase promotes recombination between two phage λ DNA molecules in an *in vitro* system. In this cross, T4 DNA topoisomerase alone is able to catalyze the recombination and produce a linear monomer recombinant DNA that can be packaged *in vitro*. ATP is not required for this recombination, while oxolinic acid stimulates it. The recombinant DNA molecules contain duplications or deletions, and the crossovers take place between nonhomologous and nonspecific sequences of λ DNA. Therefore, the recombination mediated by the T4 DNA topoisomerase is an illegitimate recombination that is similar to that mediated by *Escherichia coli* DNA gyrase. A model was proposed previously in which DNA gyrase molecules that are bound to DNA associate with each other and lead to the exchange of DNA strands through the exchange of DNA gyrase subunits. This model is also applicable to the recombination mediated by T4 DNA topoisomerase.**

DNA rearrangements are frequent in the genomes of bacteriophages, bacteria, and higher organisms. One class of rearrangements is produced by illegitimate recombination between nonhomologous sequences on two different DNA molecules or at two different sites of a DNA molecule. These events include deletion, tandem duplication, insertion, and inversion. Illegitimate recombination, especially that which results in deletion and insertion, has been studied extensively in *Escherichia coli* (see refs. 1 and 2). The crossover regions usually contain short homologous sequences that have been thought, therefore, to have a role in the recombination (3, 4). It is also known that this type of recombination takes place independently of *E. coli recA* gene function (5, 6).

We have found that *E. coli* DNA gyrase participates in illegitimate recombination in an *in vitro* system (7, 8); analysis of recombinants produced in this system does not reveal any prominent homology in crossover regions (9, 10). DNA gyrase belongs to the class of type II topoisomerases that participates in topological changes in the state of circular DNA, for example, the introduction or removal of supercoils, the formation of catenanes, and the knotting or unknotting of circular DNA (11–14). The characteristic relevant to the illegitimate recombination might be that the type II topoisomerase binds to double-stranded DNA and makes a double-stranded break (15, 16). We have proposed that two DNA gyrase molecules bound to DNA can associate with each other and cause the exchange of DNA strands through the exchange of DNA gyrase subunits (9). In fact, we have shown that some of the recombination sites where crossovers take place coincide with DNA gyrase cleavage sites, suggesting that the cleavage of DNA by DNA gyrase has an important role in the recombination process (17).

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T4 DNA topoisomerase also belongs to the group of type II enzymes (18, 19). While *E. coli* DNA gyrase and T4 DNA topoisomerase share the many common properties described above, there is the important distinction that the phage enzyme is not able to introduce negative supercoils into covalently closed circular DNA *in vitro*. Nevertheless, the T4 enzyme might be able to promote the illegitimate recombination *in vivo* as well as *in vitro*, because it has a capacity to make a transient double-stranded break in DNA (14). To determine whether the ability to introduce negative supercoils is dispensable for the illegitimate recombination, it is worthwhile to test whether T4 DNA topoisomerase can promote the recombination in an *in vitro* system.

In the present communication, I report that purified T4 DNA topoisomerase directly participates in the recombination between two λ DNA molecules. Analysis of recombinant DNA molecules indicates that the recombination mediated by T4 DNA topoisomerase is an illegitimate recombination similar to that mediated by *E. coli* DNA gyrase.

## MATERIALS AND METHODS

**Bacteria, Plasmids, and Bacteriophages.** The bacterial strains used were all derivatives of *E. coli* K12: Ymel *supF* was used for the preparation of phage samples and for the assay of total phage titers. Ymel(λ) was used for the transduction assay of recombinant phages. HI96 $Su^-$ *recA1* (λ *Dam15 FIam96B Sam7 Ram5*) (20) was used for the assay of $am^+$ plaque formers. YS1 *thr leu str minA end mal* (21) carrying a pRLM4 plasmid (22) was used for the preparation of extracts. NS428 *recA* (λ *cI857 b2 Aam11 Sam7*) and NS433 *recA* (λ *cI857 b2 Eam4 Sam7*) (23) were used for the preparation of packaging extracts. Phage λ *imm434cI Dam15 FIam96B b538 red3* and λ *imm434cI Sam7 Ram5 int6 red3* were used as parental phages in T4 DNA topoisomerase-mediated recombination (20). They are called λ*b538DF* and λ*SR*, respectively, in this paper.

**Extract and Enzyme.** Cell extracts were prepared from *E. coli* YS1 carrying pRLM4 as described (8). T4 DNA topoisomerase was kindly provided by B. Alberts (24). It is nearly homogeneous as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis.

***In Vitro* Recombination System.** *In vitro* recombination was carried out in a mixture containing T4 DNA topoisomerase, two kinds of λ DNA (50 μg/ml each), 30 mM Tris·HCl (pH 7.4), 2 mM spermidine, 1 mM ATP, 7 mM $MgCl_2$, 10 mM KCl, and 7 mM 2-mercaptoethanol. In some experiments, an extract of *E. coli* YS1 (pRLM4) (5 mg of protein/ml) was added to the mixture, which was then incubated at 28°C for 60 min, and DNA was packaged *in vitro* as described (8).

**Assay for Recombinant Phage.** Samples from crosses were plated on Ymel, for total *imm434* plaque formers and on HI96 for $am^+$ *imm434* recombinants. The frequency of recombination was defined as the fraction of $am^+$ plaque formers among total plaque formers.

**Other Methods.** Media (20), methods for sedimentation analysis (7), and restriction enzyme analysis (25) have been described.

## RESULTS

**T4 DNA Topoisomerase Promotes Recombination Between λ Phage DNAs.** To examine whether T4 DNA topoisomerase promotes recombination between two genetically marked λ phage DNAs *in vitro*, λ*b538DF* DNA and λ*SR* DNA (see Fig. 1), were incubated with varying amounts of T4 DNA topoisomerase for 60 min at 28°C. The DNA was then packaged *in vitro* and assayed for *imm434 am*$^+$ recombinant plaque formers. *b538*, a deletion of *att*, was carried by one of the parental phages to prevent site-specific recombination during *in vitro* packaging. Both phages contained *int*$^-$ and *red*$^-$ mutations to prevent homologous recombination within *recA*$^-$ indicator bacteria.

The number of total plaque formers was 56% of that of untreated DNA after incubation of λ DNA (100 μg/ml) with 25 μg of T4 topoisomerase/ml, indicating that the enzyme did not severely reduce the viability of phage DNA. The frequency of recombinant plaque formers (*imm434 am*$^+$) was not remarkable at low concentrations of topoisomerase, but increased above a concentration of about 10 μg of topoisomerase/ml and reached $1.1 \times 10^{-3}$ at 25 μg of enzyme/ml (Fig. 2). The frequency of *imm434 am*$^+$ phages was low (less than $10^{-5}$) when one of the parental phage DNA types was not added or when both parental DNAs were added only at the packaging step.

To eliminate the possibility that T4 DNA topoisomerase might act in the second step, the DNA was purified by 0.2% $NaDodSO_4$ and phenol after the first reaction, dialyzed, and then packaged. *am*$^+$ recombinant phages were again detected at a frequency comparable to that of the standard assay conditions.

The molecular nature of products formed in the recombination reaction with or without T4 DNA topoisomerase was analyzed by neutral sucrose gradient centrifugation (Fig. 3). In both samples, one major peak of [$^{32}$P]DNA and one major peak of bulk packageable DNA were found at the position corresponding to the linear monomer DNA. The [$^{32}$P]DNA sediments at a slightly slower rate than the bulk packageable DNA because half of the [$^{32}$P]DNA is λ*b538DF* that contains a large deletion and is less packageable. One major peak of *imm434 am*$^+$ recombinant DNA activity was found at the same linear monomer position in the sample from the reaction with topoisomerase, but not in the unreacted sample. This result indicates that the predominant structure of packageable recombinant DNA produced in the T4 topoisomerase-mediated reaction is a linear monomer and not a nonspecific aggregate or a collection of fragments of parental DNA. Hence, the T4 DNA topoisomerase alone is able to accomplish the recombination in the first step.

To examine the time course of recombinant formation, the reaction was interrupted at various times by adding 0.2% $NaDodSO_4$ and phenol. Recombinants started to appear within 5 min and reached a plateau at 10 min. $Mg^{2+}$ was required for the reaction, but ATP and spermidine were not. Oxolinic acid (250 μg/ml) stimulated the recombination (Table 1).

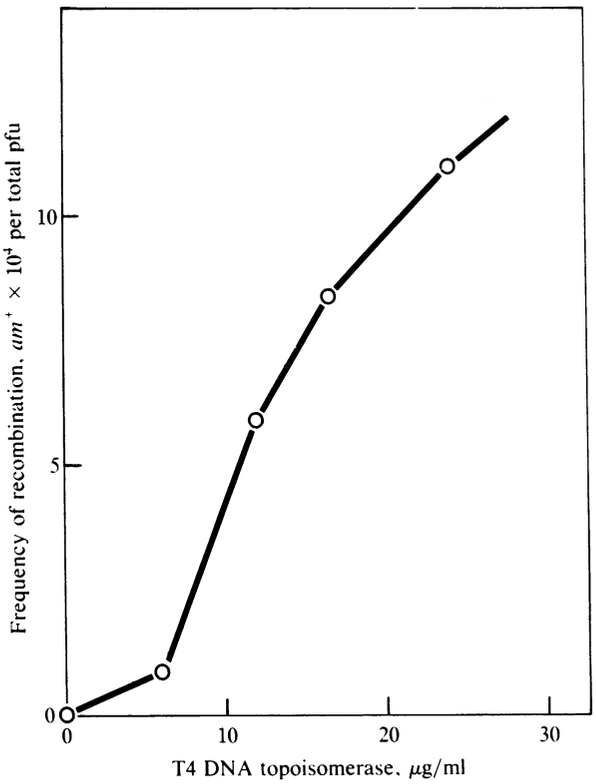


FIG. 2. Effect of T4 DNA topoisomerase on recombination. The reaction was carried out. After packaging *in vitro*, phages were assayed on *E. coli* HI96. pfu, plaque-forming units.

**Effect of Cell Extract on the Recombination.** To examine whether an *E. coli* extract stimulates the recombination in the presence of T4 DNA topoisomerase, as was the case of DNA gyrase-mediated recombination (8), the reaction was carried out in the presence of various amounts of cell extracts prepared from *E. coli* YS1 (pRLM4). This extract is active in a λ-plasmid recombination (results to be described elsewhere). pRLM4 presumably has no role in λ–λ recombination. The frequency of recombinant plaque formers increased with the amount of the extract added, to a level about 10-fold higher than that without extract (data not shown).

To examine whether the *recA* gene function from the extract plays a role in this recombination, cell extracts prepared from *E. coli* 594 *end*$^-$ (pRLM4) and its *recA1* derivative were used for recombination assays. The frequency of recombination depends on the addition of T4 DNA topoisomerase with either the *rec*$^+$ extract or *recA1* extract but is not affected by the *recA* gene mutation. This result is consistent with the conclusion that a homologous exchange

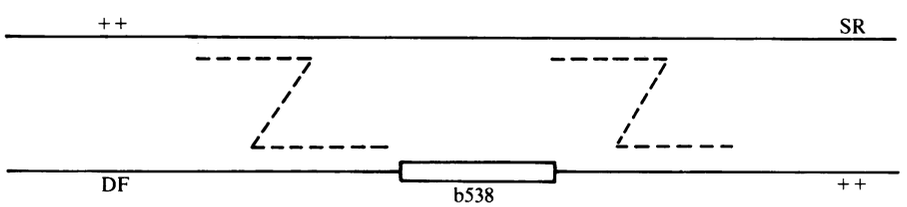


FIG. 1. Diagram of genetic maps of the parental phages showing the order and the approximate location of genetic markers relevant to the experiments. Assays on Su$^-$ *recA1* (λ *Dam15 FIam96B Sam7 Ram5*) (=HI96) measure *imm434 D*$^+$*FI*$^+$*S*$^+$*R*$^+$ recombinants. Crossovers to the right and the left of *b538* produce *b538*$^+$ and *b538* recombinants, respectively. SR, *Sam7 Ram5*; DF, *Dam15 FIam96B*.

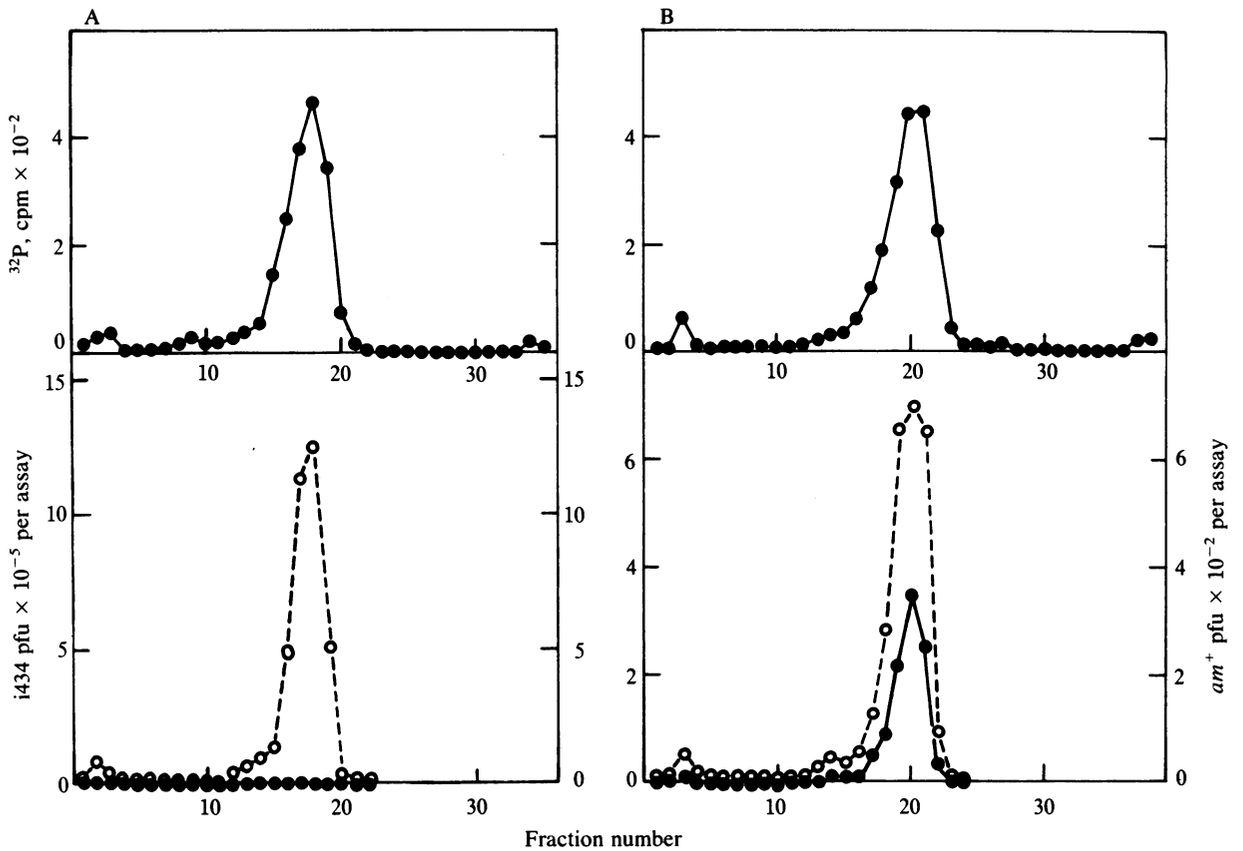


FIG. 3. Neutral sucrose gradient centrifugation analysis of the recombinant DNA. The recombination was carried out as in Fig. 2, except that the mixture contained $^{32}$P-labeled λ*SR* DNA and $^{32}$P-labeled λ*b538DF* DNA but did not contain ATP or spermidine. The reaction was terminated by the addition of 0.2% $NaDodSO_4$, and the DNA was treated with phenol and dialyzed. After heating at 65°C for 10 min, the sample was centrifuged in a 4-ml neutral sucrose gradient (5–20% sucrose in 50 mM KCl, 10 mM Tris·HCl at pH 8.0, 1 mM EDTA) at 40,000 rpm for 160 min at 15°C in a Beckman SW 60 rotor. Each fraction was assayed for $am^+$ recombinant DNA activity and for $^{32}$P radioactivity. (*A*) Reaction without topoisomerase. The input radioactivity was 4200 cpm, and about 80% of the $^{32}$P-labeled DNA was recovered. The number of fractions was 35. (*B*) Reaction with 25 μg of T4 topoisomerase/ml. The number of fractions was 38. (*Upper*) ●, $^{32}$P radioactivity. (*Lower*) ●, $am^+$ *imm434* recombinant plaque-forming units; ○, total *imm434* pfu.

between two λ DNAs is not the source of this recombination.

**Restriction Mapping of the $am^+$ Recombinant Phage.** To define the sites of joining between λ sequences, recombinant phage DNAs were digested with various restriction enzymes and the restriction endonuclease cleavage maps of the recombinants were constructed. For example, λ622, λ624, and λ625 DNAs digested with *Ava* I produced 2.3-, 2.2-, and 2.3-kilobase (kb) bands, respectively, while the parental DNAs digested with the enzyme produced a 1.9-kb band, which is located at 65.2–69.1% in λ map coordinates (Fig. 4). Other bands from the recombinants were similar to those from the parental λ*SR* DNA. Hence, the recombinants probably have small duplications or insertions in the indicated location. The rearranged regions contained no restriction sites for the seven enzymes tested in Fig. 5 that were not identifiable with sites in these regions of the parental DNA. Insertions from other parts of the λ genome are, therefore, unlikely. λ622, λ624, and λ625 had similar structures with respect to the locations of crossover points and the sizes of duplication but were distinguishable from each other by other restriction enzyme digestions, as summarized in Fig. 5.

Table 1. Conditions for formation of recombinants

| Conditions | Frequency, $am^+$ *imm434* recombinants per total *imm434* pfu |
|---|---|
| Experiment 1 | |
| Complete reaction mixture | $1.4 \times 10^{-3}$ |
| Omit spermidine | $1.1 \times 10^{-3}$ |
| Omit $Mg^{2+}$ and spermidine | $2.5 \times 10^{-5}$ |
| Omit T4 topoisomerase | $9.0 \times 10^{-6}$ |
| Experiment 2 | |
| Complete reaction mixture | $7.3 \times 10^{-4}$ |
| Omit ATP | $6.8 \times 10^{-4}$ |
| Experiment 3 | |
| Complete reaction mixture | $1.0 \times 10^{-3}$ |
| Plus oxolinic acid | $1.2 \times 10^{-2}$ |

The recombination reaction was carried out as in Fig. 2 except that the mixture contains 25 μg of T4 topoisomerase/ml. Oxolinic acid was added at a concentration of 250 μg/ml where present. The reaction mixtures were purified by 0.2% $NaDodSO_4$/phenol, dialyzed, and packaged *in vitro*. pfu, plaque-forming units.

The gel electrophoresis pattern of λ627 DNA digested with *Sca* I was similar to that of λ*SR* DNA except that a 5.5-kb band was replaced by a 3.6-kb band (Fig. 4). Hence, the recombinant has a deletion in the region between 56.2% and 67.6% of λ map coordinate. The pattern of λ628 DNA digested with *Sca* I was the same as that of λ*b538DF* DNA except that a 2.3-kb band (33.9–38.5% in λ map coordinates) was twice as dense as that of the parental DNA, and in addition a new band of 1.2 kb appeared, indicating that a duplication including the 2.3-kb segment (33.9–38.5% in λ map coordinates) was produced in the λ628 recombinant (Fig. 4). The structure of these and other recombinant phage DNAs is also shown in Fig. 5. All seven recombinant strains tested contained a duplication or a deletion in the λ genome, showing that crossovers occurred between different posi-

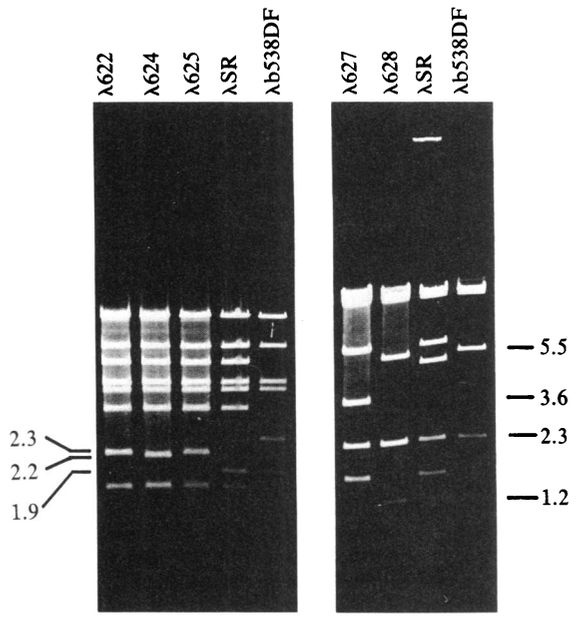


FIG. 4. Restriction analyses of the recombinant phage DNAs. (*Left*) The indicated λ DNAs were digested with *Ava* I and electrophoresed in 0.7% agarose gel. Parental λ*SR* DNA and λ*b538DF* DNA were also digested with the same enzyme and coelectrophoresed. (*Right*) The indicated λ DNAs were digested with *Sca* I and treated similarly. Molecular sizes in kb are indicated.

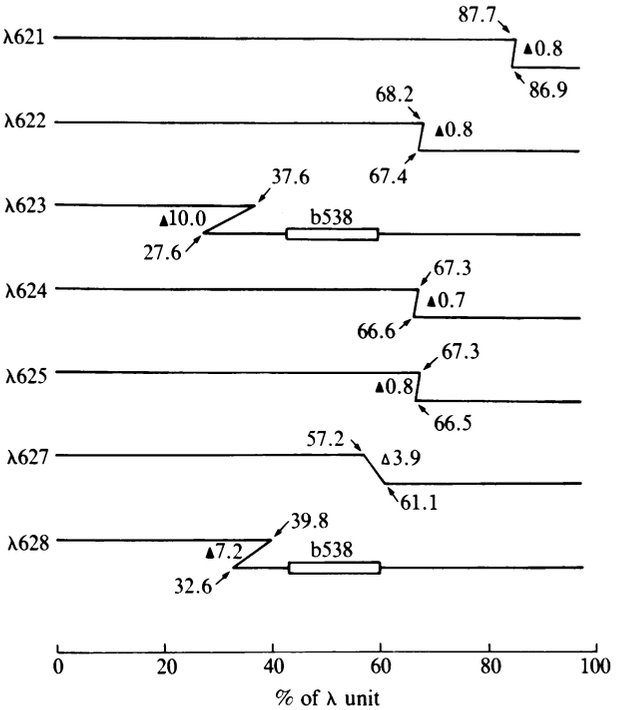


FIG. 5. Structures of the *am*$^+$ recombinant phage DNAs. The recombinant phage DNAs were digested with *Pvu* II, *Sca* I, *Hpa* I, *Ava* I, *Hin*dIII, *Mlu* I, and *Acc* I. DNAs were electrophoresed in 0.7% agarose gel, and the structures of recombinants were deduced. Crossover points are shown by arrows with numbers that give the λ map coordinate (left to right, 0 to 100). Solid triangle with its associated number, size of duplication (% of λ unit). Open triangle with its associated number, size of deletion (% of λ unit). Open rectangle, *b538* deletion originally present on parental λ *b538DF* DNA.

tions of the λ sequence, though there is a tendency that some of recombinants are localized in a central region of the λ genome. Therefore, the crossover points on λ sequence are not unique, confirming that the recombination observed here is an illegitimate recombination. However, the possibility of local homology around the crossover points is not excluded by these experiments.

## DISCUSSION

The present study indicates that T4 DNA topoisomerase promotes recombination between two phage DNA molecules. In contrast with the DNA gyrase-mediated recombination described (7, 8), *E. coli* extract is not essential here. Incubation of phage DNAs with T4 topoisomerase produces linear monomer recombinant DNA that is packageable *in vitro*. This result indicates that the product is not a nonspecific aggregate, a collection of fragments of parental DNA nor a type of intermediate DNA that carries two parental DNAs connected with each other by one or two single-stranded bridge(s). I conclude, therefore, that the enzyme alone is able to accomplish the recombination.

Structural analysis of recombinant DNA formed in the cell-free system showed that all of the recombinants between two genetically marked phage DNAs contain a deletion or a duplication at different sites on the phage genome, suggesting that the crossovers occurred between nonhomologous sequences of λ DNA. No two of the crossover sites were identical, indicating that the sites of the crossovers are neither homologous nor specific. Therefore, the recombination mediated by the T4 DNA topoisomerase is an illegitimate recombination. These properties are similar to those of the illegitimate recombination mediated by *E. coli* DNA gyrase (7). I have not yet presented any evidence on the physiological relevance of the T4 DNA topoisomerase-mediated recombination. However, I have already obtained evidence for the involvement of DNA gyrase on the *in vivo* recombination (A. Miura and H.I., unpublished result). The T4 DNA topoisomerase-mediated recombination will provide further opportunity to examine this point.

T4 DNA topoisomerase is known to catalyze the relaxation of supercoiled DNA by changing the linking number in steps of two (14). The mechanism involved in the relaxation of supercoiled DNA is a strand–passage reaction that depends on cleavage of double-stranded DNA; a product with protein covalently attached to the DNA can be formed in the presence of denaturing agent. T4 DNA topoisomerase and *E. coli* DNA gyrase share these properties. ATP is not required for the recombination reaction. This result is in contrast with that of ATP-dependent relaxation of supercoiled DNA (18), but in accord with that of ATP-independent cleavage of nonglycosylated cytosine-containing T4 DNA (26). The fact that the reaction is stimulated by oxolinic acid is also consistent with the oxolinic acid-induced cleavage of DNA by T4 topoisomerase (26). These results support the idea that the cleavage of DNA by topoisomerase has an essential role in the recombination process. A large amount of T4 topoisomerase is required for the recombination reaction as compared with that for the cleavage reaction (26). However, the ratios of enzyme to DNA in both reactions are comparable.

The purified T4 DNA topoisomerase consists of three subunits proteins of 57, 48, and 18 kDa, known to be coded by genes, 39, 52, and 60, respectively (18, 19). The native form of T4 topoisomerase seems to be a dimer (27; also see ref. 24) as is that of *E. coli* DNA gyrase. According to these physical properties of T4 DNA topoisomerase, the subunit exchange model that has been proposed for the explanation of DNA gyrase-mediated recombination is also applicable to the recombination mediated by the T4 DNA topoisomerase (see fig. 5 of ref. 8). The enzyme binds to DNA as a dimeric complex, resulting in a temporary cleavage of double-stranded DNA. One of the subunits covalently binds to each

of the 5′ termini of the DNA at the cleavage site. Two enzyme–DNA complexes then assemble into a tetrameric structure. Finally the dissociation of the tetrameric complex to dimeric complexes results in subunit exchange with accompanying exchange of DNA duplexes.

Even though the T4 topoisomerase has many properties in common with DNA gyrase, they differ from each other in one significant way. While DNA gyrase has the ability to introduce negative supercoils into circular duplex DNA substrate in an ATP-driven reaction, no such activity has been detected for the T4 topoisomerase. Based on the common features of the illegitimate recombination mediated by both enzymes, one can conclude that the supercoiling activity is not essential for this type of recombination.

Eukaryotic type II DNA topoisomerases have been isolated from yeast, *Drosophila*, *Xenopus*, and mammalian cells. These eukaryotic type II enzymes resemble the T4 topoisomerases in many properties including their lack of ability to introduce negative supercoils into circular duplex DNA. One can imagine that eukaryotic topoisomerases may promote the illegitimate recombination that has been often observed in eukaryotic cells. For example, simian virus 40 DNA is integrated into or excised from the host chromosome by an illegitimate recombination (28). Similarly, DNA transformed into eukaryotic cells undergoes a variety of rearrangements such as deletion or insertion (29, 30). Our *in vitro* system may make it possible to develop a new approach to study illegitimate recombination in eukaryotic cells.

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