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Illegitimate recombination mediated *in vitro* by DNA gyrase of *Escherichia coli*: Structure of recombinant DNA molecules

(type II topoisomerase/oxolinic acid/insertion/deletion/junction sequence)

HIDEO IKEDA, KAZUKO AOKI, AND AYAKO NAITO

The Institute of Medical Science, The University of Tokyo, P.O. Takanawa, Tokyo 108, Japan

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ABSTRACT **We have developed a cell-free system from *Escherichia coli* for studying illegitimate recombination between nonhomologous DNA molecules. The recombination is stimulated by oxolinic acid, an inhibitor of DNA gyrase. The stimulation is abolished by coumermycin A_1 and is not found in extracts of nalidixic acid-resistant (*gyrA*) mutants. We therefore inferred that DNA gyrase directly participates in illegitimate recombination, at least in the presence of oxolinic acid [Ikeda, H., Moriya, K. & Matsumoto, T. (1981) *Cold Spring Harbor Symp. Quant. Biol.* 45, 399–408]. The structure of recombinant DNA molecules formed in the presence of oxolinic acid from a cross between phage λ and plasmid pBR322 DNAs was analyzed by heteroduplex mapping. Among nine isolates tested, two recombinants were formed by the insertion of the plasmid into the λ genome. The seven other recombinants had more complicated genome structures. Insertion of pBR322 was accompanied by a deletion on one of the genomes. In all cases, the end points of deletions coincided with one end of the pBR322 insertion. Recombination sites seemed to be distributed randomly on the λ and pBR322 genomes. Analysis of nucleotide sequences of the recombination junctions proved that the crossover took place between nonhomologous DNA sequences. A model for DNA gyrase-mediated illegitimate recombination is discussed.**

Chromosomal rearrangements take place throughout the genomes of bacteriophages, bacteria, and higher organisms. Tandem duplication appears to be produced by illegitimate recombination between nonhomologous sequences originally present on different DNA molecules (1). Abnormal excision of λ prophage from the bacterial chromosome results in the formation of specialized transducing phage such as λgal and λbio (2). These recombinations are generally thought to be recombination between nonhomologous regions or very short homologous regions of DNA. The illegitimate recombination observed in the *Escherichia coli* system usually takes place independently of bacterial *recA* function and viral *int*, *xis*, and *red* functions (1, 3–6).

We have been working to develop a cell-free illegitimate recombination system of *E. coli* and have recently shown that an *in vitro* packaging system consisting of lysates of induced lysogens is capable of recombining two heterologous DNA species (7). The packaging mixtures contain a large amount of concatemeric λ DNA derived from prophage. This λ DNA is a good substrate for illegitimate recombination as well as packaging. When a plasmid DNA is incubated with the packaging mixture, the plasmid recombines with the λ DNA and is packaged into a λ head, resulting in the formation of recombinant phages. Studies with this system showed that oxolinic acid, an inhibitor of DNA gyrase, stimulates recombination, and the stimulation is abolished by coumermycin A_1, another type of gyrase inhibitor. We therefore inferred that DNA gyrase directly participates in this recombination (7). In the present paper, we have studied the structure of λ–pBR322 recombinant DNAs formed *in vitro* in the presence of oxolinic acid. The results show that the recombinants were produced by reciprocal recombination with or without accompanying deletions in λ or plasmid DNA. The analysis of the nucleotide sequences of recombination junctions proved that the recombination occurs between nonhomologous DNA sequences.

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MATERIALS AND METHODS

Bacteria, Phages, and Plasmids. Bacterial strains are all derivatives of *E. coli* K-12: 594 *sup*^{0} (8); HI225 *recA1* (9); HI25 = 594 (λ *cI857 Eam4 Sam7*) (8); HI26 = 594 (λ *cI857 Dam15 Flam96B Sam7*) (8); HI507 = HI225 (λ *cI857 Eam4 Sam7 int6 red3*) (7); HI501 = HI225 (λ *cI857 Dam15 Flam96B Sam7 int6 red3*) (7); HI529 = 594 (λ *cI857 Aam11 Sam7 xis6 b515 b519*) (7); HI530 = 594 (λ *cI857 Eam4 Sam7 xis6 b515 b519*) (7); Km960 [pBR322], provided by K. Matsubara; AE483 = C600 [pAO3] (10); KS2662 = Hfr H *gal–bio* deletion *guaA–guaB* deletion [pKY2662]. pKY2662 is a cosmid carrying ColE1 derivatives and λ *Eco*RI-*cos-Hpa* I fragment (5.3 megadaltons) (11). λ *cI857 Dam15 Flam96B Sam7 red3 b538* and λ*cI857 Eam4 Sam7 red3 b538* were used for preparation of a packaging mixture. λ *imm434* was used for heteroduplex analysis.

Media, Buffer, and Reagents. Media and buffers were described previously (7, 8). The source and final concentrations of inhibitors used are as follows: ampicillin, 20 μg/ml (Banyu Pharmaceutical, Tokyo, Japan); tetracycline (Sigma), 12 μg/ml; oxolinic acid (provided by J. Tomizawa), 50 μg/ml.

***In Vitro* Recombination–Packaging System.** A packaging mixture (25 μl per tube) was prepared by heat induction of λ lysogens of *E. coli* K-12, HI25, and HI26, as described (8). Packaging mixtures from λ *int*$^-$ *red*$^-$ lysogens, HI507 and HI501, and those from λ *xis*$^-$ *b515 b519* lysogens, HI529 and HI530, were prepared by superinfection with the same phages as prophage after heat induction (7). A packaging mixture containing λ *b538* DNA was prepared by infection of λ *cI857 Dam15 Flam96B Sam7 red3 b538* and λ *cI857 Eam4 Sam7 red3 b538* into *E. coli* 594 (7).

For *in vitro* recombination, pBR322 DNA (1 μg in 10 μl) carrying the ampicillin resistance (Ap^r) determinant was incubated in the packaging mixture as described (7).

Nucleic Acid Analysis. Restriction endonuclease analyses were accomplished by standard procedures (12). Heteroduplex analysis was as described (7). Measured lengths of DNA were calibrated by the following intramolecular markers (described in percent of λ DNA length): *b519* deletion (40.6–47.6), *b515* deletion (49.3–53.4), *b538* deletion (43.0–60.1), *imm434* sub-

Abbreviations: bp, base pair(s); kb, kilobase(s); Ap^r, ampicillin resistance; Tet^r, tetracycline resistance.

stitution (73.5–79.1) (13). The sequences of recombination junctions of the recombinant phage λ296 were determined by the terminal labeling method of Maxam and Gilbert (14).

RESULTS

Stimulation of Illegitimate Recombination by Oxolinic Acid. When pBR322 DNA (1.0 μg per tube) was mixed in the absence of oxolinic acid with the packaging mixture prepared from *rec*$^+$ lysogens containing a normal size λ prophage, the number of plaque formers was about 10^9 per tube (35 μl) and Ap^r transducing phages were found at a frequency of 1.3×10^{-7} per plaque-forming unit. When oxolinic acid (50 μg/ml) was added to the packaging mixture together with pBR322, the number of plaque formers did not change; however, the frequency of the Ap^r transducers was about 9-fold higher than that in the absence of the drug.

In three other experiments, we examined this oxolinic acid-induced recombination in packaging mixtures defective in bacterial *recA* function or viral recombination functions. Oxolinic acid stimulates the recombination in a *recA*$^-$ *int*$^-$ *red*$^-$ packaging mixture, in a *b515 b519 xis*$^-$ packaging mixture, and in a *b538* (*int* deletion) *red*$^-$ packaging mixture to extents similar to the extent in a *rec*$^+$ *int*$^+$ *red*$^+$ *xis*$^+$ mixture (data not shown). These results showed that the *recA*, *int*, *red*, and *xis* gene products are not necessary for the illegitimate recombination induced by oxolinic acid.

We conclude that the stimulation of recombination by oxolinic acid is reproducible in various packaging systems, and we therefore refer to the recombination occurring in the presence of the drug as "DNA gyrase-mediated recombination."

Heteroduplex Mapping of the λ *b538*–pBR322 Recombinant Phages Produced by DNA Gyrase-Mediated Recombination. The structure of the recombinant DNA generated *in vitro* by DNA gyrase-mediated recombination was examined by isolating Ap^r transductants formed in the presence of oxolinic acid from the cross between λ *b538* and pBR322. The transductants were grown and induced by heating at 42°C after superinfection with λ *cI857 Sam7* helper phage. These lysates were tested for frequencies of Ap^r phages. Among 32 Ap^r transductants tested, 28 produce high-frequency-transducing (HFT) lysates in which the Ap^r transducers were 10^{-1} to 10^{-2}. We further purified nine Ap^r phages by CsCl density gradient centrifugation and analyzed their DNA by heteroduplex mapping.

In heteroduplex molecules between λ317 recombinant DNA and λ *imm434* DNA, an insertion loop was observed in addition to the *b538* loop and *imm434* loop. The length (±SEM) of the single-stranded loop is 9.1 ± 0.3% of a λ unit, which corresponds to the size of pBR322. Heteroduplex analysis with λ322 recombinant DNA showed an insertion loop corresponding to pBR322, though the point of insertion is different from that in the preceding molecule. Hence these two recombinant DNAs were formed by insertion of pBR322 monomer into λ *b538* DNA (Fig. 1).

Heteroduplex molecules between λ321 and λ *imm434* DNA contained a substitution loop. One of the single-stranded parts in the loop had a length of 8.4 ± 0.4% of a λ unit, which corresponds to the size of pBR322. Another single-stranded part had a length of 3.8 ± 0.3% of a λ unit, which presumably is a deletion of the λ genome. Analyses of heteroduplex molecules of recombinants (λ291, λ296, and λ323) showed essentially the same results, except that λ291 contains an insertion of a pBR322 dimer with a deletion of a λ segment (Fig. 1).

Heteroduplex molecules between λ319 and λ *imm434* DNA contained an insertion loop. The length of the loop is 12.0 ± 0.6% of a λ unit, which is 1.4-fold that of pBR322. We presume

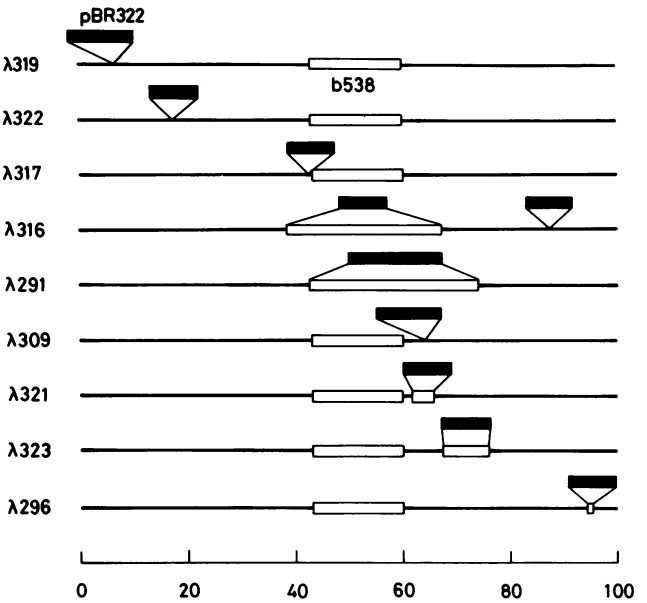

FIG. 1. Physical maps of nine λ *b538*–pBR322 recombinant DNAs characterized by heteroduplex mapping. Lengths of DNA segments were calculated on the basis of the following numbers: λ DNA, 49.5 kilobases (kb) (15); pBR322, 4.36 kb (16). On the scale (percent) 0 and 100 represent the left and right cohesive ends of λ phage, respectively. Lines represent λ DNA; open rectangles represent λ deletion; solid rectangles represent pBR322.

that this recombinant DNA was formed by an insertion of pBR322 dimer with a concomitant deletion of pBR322 DNA. In heteroduplex analysis of λ319 with pBR322 DNA, a deletion loop would not be observed on the pBR322 duplex if the deletion is located at the end of the pBR322 segment but not inside of it. As expected, no deletion loop was found on the pBR322 duplex loops of 14 heteroduplex molecules. This result suggested that a deletion of the pBR322 segment is located at the site of the junction between λ and pBR322 (Fig. 1). λ309 was also classified into the same group (Fig. 1).

The λ316 recombinant contained two pBR322 segments which were inserted and substituted simultaneously at different positions in the λ DNA (Fig. 1).

We therefore conclude that the recombinant DNA was generated in the presence of oxolinic acid by an insertion or substitution of pBR322 into λ DNA. The substitution recombinants contain a deletion of either λ or pBR322 DNA at the point of insertion. The recombination junctions are distributed randomly over the λ genome.

Cloning of Recombination Junctions. In order to determine the location and structure of the sites of recombination on pBR322, we have cloned the recombination junctions of several λ *b538*–pBR322 recombinants. A recombinant, λ296, has a pBR322 insertion near the *R* gene of the λ genome (Fig. 1) and shows an Ap^r and tetracycline resistance (Tet^r) phenotype. From this recombinant, a *Cla* I restriction fragment that contains the Ap^r gene and the *ori* site of pBR322 but not the Tet^r gene was isolated as a plasmid. The *Eco*RI fragment that has the Tet^r gene but not the Ap^r gene was inserted into plasmid pAO3 that had been cleaved with *Eco*RI. The plasmids were named pHI40 and pHI18, respectively. They were analyzed by restriction enzymes and the physical maps were constructed as shown in Fig. 2. The results show that they contain recombination junctions. The nucleotide sequences of the junctions will be described in the next section.

From other Ap^r recombinants, λ235, λ317, λ321, λ322, and λ323, *Eco*RI fragments that have the Ap^r gene and the *ori* site

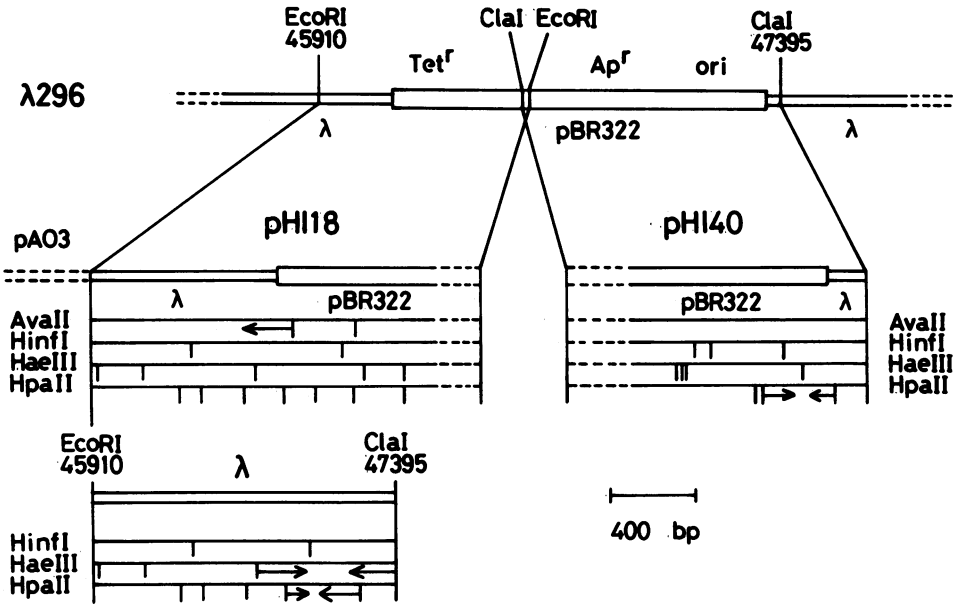

FIG. 2. Restriction enzyme maps of pHI18, pHI40, and 1.4-kb λ *Eco*RI–*Cla* I fragment and sequencing strategy employed to locate the recombination junctions. pHI18 and pHI40 were derived from λ296 recombinant DNA and contain recombination junctions that are located between the *Ava* II site [base pair (bp) 1,759] and the *Hae* III site (bp 1,948) of pBR322 (see text). The 1.4-kb λ fragment (bp 45,910–bp 47,375) was isolated by the cleavage of pKY2662 by *Eco*RI and *Cla* I restriction endonucleases. The arrows below each restriction map show the positions and the directions in which the sequences were determined. The nucleotides are numbered according to a tentative λ map coordinate defined by F. R. Blattner, D. L. Daniels, and J. L. Schroeder (personal communication).

of pBR322 were ligated and isolated as Ap^r plasmids. Another Ap^r Tet^r recombinant, λ233, was cleaved by *Eco*RI and the *Eco*RI fragment containing the Tet^r gene was inserted into plasmid pAO3 that also had been cleaved by *Eco*RI. The sites of the recombination junctions were mapped by restriction enzymes and are shown in Fig. 3. The results show that the recombination sites are located at various positions between the *Bam*HI site and the *Pvu* II site of pBR322. The localization of the insertion sites to the right side of the circular pBR322 map (Fig. 3) can be explained by the requirement of the recombinant phages for an intact Ap^r gene because of their selection by ampicillin resistance. The recombinant phages also must contain the *ori* site because of the lack of the *att*λ site (except λ233 and λ235). There seems to be no preferential recombination site on the pBR322 genome.

Nucleotide Sequences of the Junctions of a Recombinant DNA. The plasmids for sequencing are pHI18 and pHI40, which contain the left-hand and right-hand junctions of the λ296 recombinant. The 1,060-bp *Ava* II fragment from pHI18 and the 430-bp *Hpa* II fragment from pHI40 contain the junctions (see Fig. 2). These fragments were end-labeled and their sequences were determined by the procedure of Maxam and Gilbert (14). We also determined the sequence of a part of parental λ DNA corresponding to the recombination sites (see Fig. 2).

Fig. 4*a* shows the sequences of the left- and right-hand junctions that are found in pHI18 and pHI40, respectively. The sequences of the λ DNA segment corresponding to the recombination sites and that of pBR322 are also shown (Fig. 4 *b* and *c*). The structure of the λ296 recombinant DNA can be described as follows: There is a long stretch of λ DNA sequences from the left cohesive end to bp 46,786. pBR322 sequences then begin at bp 1,823, stretching in an apparently complete genome unit to bp 1,824, where a stretch of λ DNA sequences again occurs from bp 47,096 to the right-hand cohesive end (Fig. 4*d*). A deletion of a 309-bp λ segment has therefore been detected at the site of the pBR322 insertion. The result shows that the recombination sites of parental λ and pBR322 DNA have no homologous sequence. The scheme obtained in Fig. 4 is consistent with the result of heteroduplex mapping of λ296 DNA.

DISCUSSION

We have previously shown that oxolinic acid stimulates illegitimate recombination between λ and pBR322 DNAs in an *E. coli* cell-free system and the stimulation is abolished by coumermycin (7). This result suggests that DNA gyrase directly affects the recombination reaction but does not indirectly affect it by the activity of supercoiling, because both drugs exert inhibitory effects on the supercoiling of DNA (17, 18). One can hypothesize that these drugs affect the recombination by activities not relating to their effect on DNA gyrase. However, another line of evidence, that the effects of oxolinic acid and coumermycin on the recombination are not observed in the nalidixic acid-resistant (*gyrA*) and coumermycin-resistant (*gyrB*) packaging systems, respectively, leads to the conclusion that the targets of these drugs are gyrase A and B proteins. Furthermore, we have observed that a *gyrB* mutation affects the recombination between λ and pBR322 DNAs *in vivo* (to be published elsewhere).

In the present study, recombinant phages formed in the cell-free system in the presence of oxolinic acid were isolated and analyzed by heteroduplex mapping. We found two types of recombinant DNA, insertion type and substitution type. The former is composed of entire λ and pBR322 genomes, suggesting that they were formed by a single reciprocal crossover. The latter class of recombinants has a deletion of a part of λ or pBR322 genome at the recombination junctions. The mechanism of the formation of the substitution-type recombinants will be discussed in the last part of this section.

DNA gyrase cleaves pBR322 DNA at two preferred sites (bp 989 and bp 2,608) (ref. 19; M. Gellert, personal communication). If the cleavage of DNA by gyrase plays a role in the initiation of strand exchange, it is expected that the recombination sites of pBR322 DNA are localized on these sites. The analyses

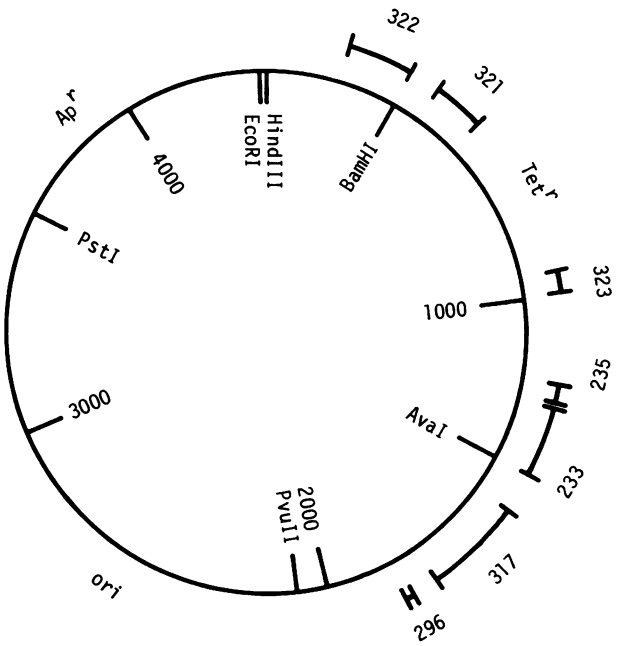

FIG. 3. Distribution of recombination junctions of seven λ–pBR322 recombinants on the pBR322 genome. Physical maps of λ296, λ317, λ321, λ322, and λ323 were shown in Fig. 1. λ233 and λ235 were obtained from the cross between λ *b515 b519* and pBR322 (in the presence of oxolinic acid). As determined by heteroduplex mapping, these recombinants were formed by the substitution of pBR322 dimer. *Eco*RI fragments that have recombination junctions were cloned by isolating Tet^r or Ap^r transformants after ligation. The locations of the junctions were determined by restriction enzyme mapping. The plasmid pBR322 map is based on the result of Sutcliffe (16). The recombination junctions are located in the region shown by bars outside the circular pBR322 map. The regions where the junctions exist are as follows: λ233, *Hinf*I (bp 1,303)–*Ava* II (1,438); λ235, *Hpa* II (1,283)–*Hinf*I (1,303); λ317, *Hinf*I (1,524)–*Ava* II (1,759); λ321, *Hae* III (400)–*Hpa* II (533); λ322, *Hae* III (173)–*Bam*HI (375); and λ323, *Hae* III (939)–*Hae* III (990). The junction of λ296 was located at bp 1,823 as will be described in Fig. 4.

of the recombinant DNAs obtained in our system showed, however, that the recombination sites are widely scattered on the pBR322 genome. DNA gyrase in this recombination system may have a substrate specificity different from that of the cleavage system of Gellert *et al.* (19) because of interactions of other proteins with DNA gyrase or DNA itself in our unpurified system.

We have determined the nucleotide sequences of the junctions of a recombinant DNA, λ296. The result showed that there was no homologous sequence between the sequences of recombination sites of parental λ and pBR322 DNA. Furthermore, it is interesting to compare the sequences of sites of gyrase-mediated cleavage with those of the recombination sites. Morrison *et al.* (20) found a very short sequence, T-G, at the sites of gyrase-mediated cleavage, whereas Gellert *et al.* (19) did not recognize any common sequence in the cleavage sites. We have found no common sequence between the recombination site and these gyrase-mediated cleavage sites so far.

We have proposed a model for illegitimate recombination in which the gyrase–DNA complex plays an essential role in the joining reaction between DNAs (7). DNA gyrase binds to DNA as an A_2B_2 complex, in which A and B are coded by *gyrA* and *gyrB*, respectively (21). Upon binding of gyrase to DNA, gyrase transiently cleaves double strands of DNA, resulting in an intermediate structure in which each *gyrA* protomer covalently binds to each of the 5′ termini of the DNA at the cleavage site (Fig. 5*b*). The gyrase–DNA complex containing two AB subunits then assembles with another gyrase–DNA complex, forming a tetrameric structure (A_4B_4) (Fig. 5*c*). We assume that the interactions between the four subunits are isologous, so that the dissociation of the tetrameric form to dimeric forms results in the subunit exchange (Fig. 5*d*). A typical example of the dimer–tetramer equilibrium has been shown in the case of concanavalin A, one of a group of plant proteins termed lectins (22–24). The double-stranded breaks are then sealed upon release of gyrase from DNA, leading to the formation of recombinant DNA. Oxolinic acid presumably has a role in the accumulation of the recombinogenic structures by increasing the half life of the gyrase–DNA complex.

The fact that the four base-staggered ends created by gyrase have many different sequences raises the question that mismatched ends would have difficulty in rejoining themselves. Nevertheless, we can imagine that rejoining between the mismatched ends would be enforced by the putative activities of subunit exchange and resealing harbored on gyrase. Recom-

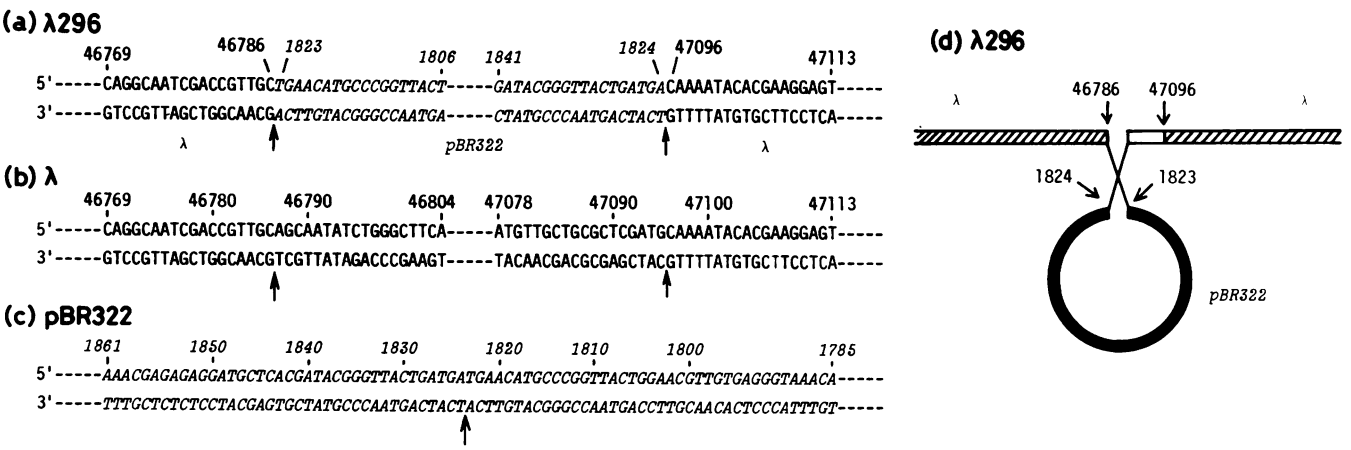

FIG. 4. Nucleotide sequences at the λ-pBR322 junctions of λ296 recombinant DNA. (*a*) Sequences at the ends of the inserted pBR322. The sequence of pBR322 is shown in italics. Vertical arrows indicate the recombination junctions. (*b*) Sequences of the parental λ DNA. The sequences shown here are based on the work of F. Sanger, A. R. Coulson, and G.-F. Hons (personal communication) and our work. Numbers are based on the numbering system of F. R. Blattner, D. L. Daniels, and J. L. Schroeder for the λ DNA sequence (personal communication). (*c*) Sequence of the parental pBR322 DNA, known from the work of Sutcliffe (16). In *b* and *c*, vertical arrows indicate the sites of recombination on the parental DNAs. (*d*) Schematic representation of the structure of the λ296 recombinant DNA. The hatched rectangles represent λ DNA; the open rectangle represents the deletion of λ DNA; the thick loop represents pBR322.

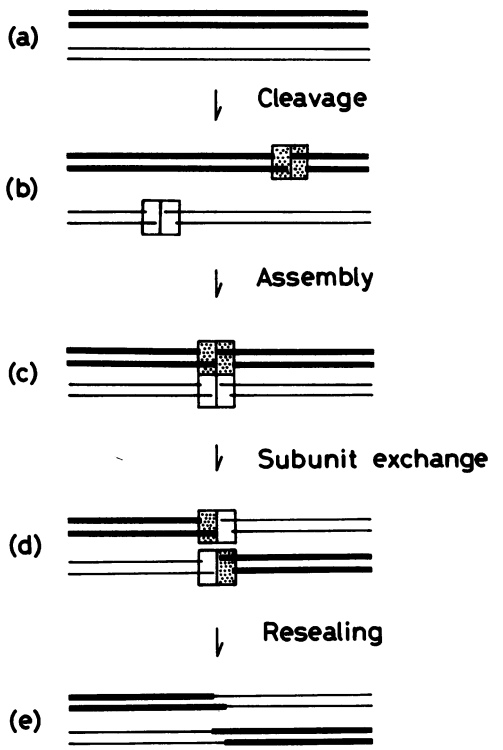

FIG. 5. Gyrase subunit exchange model for illegitimate recombination. Each rectangle represents the AB subunit of DNA gyrase, composed of one gyrase A protomer and one gyrase B protomer. Two combined rectangles constitute the AB–AB form, which is a putative gyrase structure. The gyrase–DNA complex (*b*) assembles with another gyrase–DNA complex, forming the tetrameric structure A_4B_4 (*c*). The dissociation of the tetrameric form to dimeric forms results in the subunit exchange that leads to the exchange of DNA strands (*d*).

binant DNA molecules containing the mismatched bases might be repaired before packaging or directly packaged without repair. Our sequencing data are consistent with the latter possibility because no trace of the mismatch correction was observed.

Heteroduplex analyses of the recombinant DNA revealed the frequent occurrence of substitution recombinants. This phenomenon can be interpreted in two ways. One explanation is that an intermediate of an insertional recombination carries a covalently bound gyrase molecule at each end of the insertion. These enzymes still have an ability to induce the subunit exchange with other gyrase molecules bound to other sites of DNA, leading to the formation of an additional deletion on the λ or pBR322 genomes. A second explanation is that first crossover takes place in a nonreciprocal way, resulting in the generation of linear recombinant DNA molecules. Then the two free ends are bound to each other by a joining reaction. Nibbling of the free ends by an exonuclease can occur before the joining reaction, leading to the formation of substitution-type recombinant DNA. We favor the former explanation for two reasons. First, the deletions would be generated simultaneously in both the λ and pBR322 genomes if the latter explanation is correct. Second, the joining reaction between two free ends would not take place so efficiently because linear pBR322 is a less efficient substrate than circular pBR322 for the recombination (unpublished result). Further critical experiments are necessary to clarify this point.

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