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Illegitimate recombination at the replication origin of bacteriophage M13

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ABSTRACT **Hybrids composed of phage M13 and plasmid pHV33 were used to study the formation of deletions in *Escherichia coli*. Eighty to ninety percent of the deletion endpoints were at the position of the nick introduced into the M13 replication origin by the phage gene II protein. This suggests the existence of a novel mechanism of illegitimate recombination.**

Recombination between sequences with little or no homology was termed "illegitimate" by Franklin (1), who proposed two classes of models to illustrate that process. The first involves errors of DNA replication, and the second, errors of enzymes that cut and join DNA molecules. They are conceptually related to copy-choice and breakage-and-reunion models of homologous recombination, respectively. Several studies supporting models of one or the other class were reported.

Spontaneous deletions usually occur in the *Escherichia coli lac* operon between short repeated sequences. This led Miller and coworkers (2) to suggest that deletions are generated by "slippage" of the replication machinery from one repeat to the other without copying the sequences between repeats. Deletions of *in vitro*-constructed palindromic sequences have been suggested to result from a similar process (3). The observation that naturally existing palindromic sequences are also often deleted has been interpreted as lending support to the slippage model (4).

DNA gyrase (topoisomerase type II, EC 5.99.1.3) was found to affect illegitimate recombination between bacteriophage λ and plasmid pBR322 in *E. coli* cell-free extracts. One explanation proposed is that a gyrase molecule, which is composed of two subunits, first cleaves and remains transitorily bound to the two strands of a DNA molecule. Next, by error, it exchanges one of the subunits with a gyrase molecule interacting with another genome. Finally, it joins the cleaved strands and thus generates a recombination intermediate (5, 6).

Studies of the properties of purified eukaryote DNA topoisomerases I (EC 5.99.1.2) have identified yet another possible mechanism of illegitimate recombination (7, 8). These enzymes cleave single-stranded DNA in regions with the potential for base-pairing (9) and remain bound to the generated 3'-phosphoryl end. In a subsequent reaction they can join this end to a 5'-hydroxyl end of single- (7) or double-stranded (8) DNA molecules. Resemblance found between the topoisomerase recognition sites (10) and the short sequences that recombined during excision of simian virus 40 provirus from a rat cell line genome suggested that topoisomerases may be involved in illegitimate recombination *in vivo* (11).

We show here that deletions are generated with high frequency by joining of the nucleotide immediately adjacent to the nick introduced into the replication origin of the *E. coli* phage M13 by the phage gene II protein (12) to a nucleotide elsewhere in the genome. This may be an example of a novel breakage and reunion mechanism of illegitimate recombination.

MATERIALS AND METHODS

The bacterial strains used were *Bacillus subtilis* HVS49 (*trpC2 hisA aroB2 tyrA*; ref. 13) and *E. coli* GY2098 (HfrH, *hsdR leu*; R. Devoret, Centre National de la Recherche Scientifique, Gif sur Yvette), HVC746 (HfrH, *hsdR leu*$^+$ spontaneous revertant of GY2098), D162 (*thy lac leu rep-3*, Su$^-$, ref. 14), and AB1157 (*thrA1 leu-6 thi-1 proA2 his-4 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpl-31 supE44*; J. Clark, University of California, Berkeley). The Rec$^-$ strains were all isogenic to AB1157. They were rendered *hsdR* by conjugation with GY2098 (HfrH) after cotransfer of *hsdR* and *thr*$^+$. *recA* strains JC5495 and JC5547 were rendered Rec$^+$ prior to conjugation by introduction of a RecA$^+$ λ phage. This phage was eliminated from the exconjugants by segregation. All the Rec$^-$ strains harbored F'*lacI*^{q}*Z* M15 *pro*$^+$ plasmid (15). The Rec$^-$ phenotype was routinely tested by sensitivity to ultraviolet light. The Rep$^-$ strain D162 was rendered *hsdR* by conjugation with a *leu*$^+$ revertant of GY2098 after cotransfer of *hsdR* and *leu*$^+$ markers. It harbored pOXKm plasmid (M. Chandler, Toulouse University).

The plasmid pHV33 is composed of pBR322 and pC194 (16–18). The plasmids pHV672 (19) and pHV673 were constructed by joining *Eco*RI-cleaved M13 mp2 phage (20) to pHV33; they were isolated in *B. subtilis*. pHV698 was constructed by replacing the small pBR322 *Cla* I–*Eco*RI fragment with the 540-base-pair (bp) M13 mp2 *Cla* I–*Eco*RI fragment that carries the phage replication origin. pHV702 was obtained by inserting pC194 into the *Hin*dIII site of pHV698. R199 and R229 are two f1 derivatives (21, 22), R208 is composed of R229 linked to pBR322 (21).

Nucleotide coordinates used for pBR322 are from Sutcliffe (23) as modified by Backman and Boyer (24); for M13, from van Wezenbeek *et al.* (25); and for pC194, from Horinouchi and Weisblum (26), corrected and numbered from the *Hin*dIII site (27). The *lac* region of M13 mp2 was numbered 1–789 in the direction of transcription of the operon.

Plasmid DNA was extracted from *B. subtilis* and from *E. coli* by the clear-lysate method (28, 29) and was purified by chromatography on hydroxyapatite columns (30). For analytical purposes, plasmid DNA was extracted from 1.5-ml overnight cultures (31). The terminal-labeling method was used for DNA sequencing (32). Transformation of *B. subtilis* and *E. coli* competent cells and plasmid transduction have been described (28, 33, 34).

RESULTS

Isolation of Deletion Plasmids. Plasmid pHV33, composed of the *E. coli* plasmid pBR322 and the *Staphylococcus aureus* plasmid pC194, is viable in *E. coli* (18). We have joined the

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Abbreviations: bp, base pair(s); kb, kilobase(s).

phage M13 mp2 to pHV33 and failed to isolate hybrids of the correct structure in *E. coli*. A multitude of smaller plasmids, which could have been generated by deletions, were obtained instead. This suggested that hybrids between pHV33 and M13 mp2 were not viable in *E. coli* and that they could be used to study the formation of deletion mutants in this organism.

We isolated plasmids composed of pHV33 and M13 mp2 in *B. subtilis* and named them pHV672 and pHV673 (Fig. 1). The two plasmids, which differ in the relative orientation of the parental genomes, were used to transform *E. coli* restrictionless (*hsdR*), competent cells to ampicillin- or tetracycline-resistance (Ap^R or Tc^R). The chloramphenicol-resistance gene of pHV33 is not expressed efficiently in *E. coli* and was therefore not used in selections. pHV672 transformed 1–2% as efficiently as pHV33, whereas pHV673 transformed 0.01–0.02% as efficiently (Table 1). Plasmids were extracted from 70 independent transformants obtained using pHV672 and from 30 obtained using pHV673 and were analyzed by gel electrophoresis. They ranged from 3.1 to 7.2 kilobases (kb) in size in the case of the pHV672 transformants and from 7.2 to 8.2 kb in the case of the pHV673 transformants. This indicates that they were generated from the 14-kb parental plasmids by deletions 6–11 kb long and that only plasmids in which deletions took place were viable. To demonstrate that deletions occurred in *E. coli* rather than in *B. subtilis*, we purified pHV672 monomers by gel electrophoresis and used them to transform *E. coli*. All the transformants contained plasmids having undergone a deletion.

Assuming that initially equal numbers of cells were transformed per ng of DNA for pHV672, pHV673, and pHV33, the frequency of deletions occurring in *E. coli* may be estimated by comparing the subsequent recovery of transformants (i.e., the transforming efficiency of pHV672 and pHV673 with that of pHV33; Table 1). This comparison suggests that deletions occurred in at least 2% of pHV672 molecules, making this a frequent event, whilst in the case of pHV673 deletions occurred 1/100th as often. It may be that deletions occurred that resulted in nonviable plasmids; this formal possibility was not investigated.

Phenotypes of the *E. coli* transformants were determined by replica-plating between 10^2 and 10^3 colonies obtained with each plasmid (Table 1). Only a limited number of phenotypic classes were observed (three for pHV672 and one for pHV673; a maximum of five classes could have been detected for each plasmid). This indicates that deletions were not eliminating segments from the two plasmids at random.

Restriction Mapping of Deletions. We extracted plasmid DNA from 21 clones obtained with pHV672 and 8 with pHV673, such that each observed phenotype (Table 1) was represented at least seven times. More than one plasmid was often observed in these samples. To obtain pure plasmid species, we retransformed *E. coli* and used a single transformant, matching the phenotype of the donor clone for further analysis. Each transformant contained a unique plasmid species, as judged by gel electrophoresis. Nine of the 21 plasmids derived from pHV672 could be allocated to one or the other of two narrow size classes: 5 of 11 Ap^R plasmids were 3090–3190 bp long, while 4 of 7 Ap^R Tc^R plasmids were 6300 bp long. The remaining 12 plasmids were all of different sizes. Six of the 8 plasmids derived from pHV673 were 8000–9000 bp long, while 2 were 7200 bp long. These results suggested that deletions might occur at nonrandom sites and thus support the hypothesis based on the genetic analysis (see above).

Table 1. Transforming efficiencies and phenotypes observed with plasmid DNAs

Plasmid	Selection	Transformants, no. per ng of DNA	Phenotype Ap	Tc	Cm	Proportion
pHV33	Ap	5000	R	R	R	1.0
	Tc	5000	R	R	R	1.0
pHV672	Ap	100	R	S	S	0.4
			R	R	S	0.5
			R	R	R	0.1
	Tc	50	R	R	S	0.4
			R	R	R	0.6
pHV673	Ap	1	R	R	R	1.0
	Tc	0.5	R	R	R	1.0
pHV672H	Ap	0.4*	R	R	R	1.0
pHV672H + mp2	Ap	20*	R	S	S	0.6
			R	R	S	0.4

R, resistant; S, sensitive.
*Average values obtained for pHV672H1 and pHV672H2.

Nineteen plasmids derived from pHV672 were subjected to detailed restriction analysis. In 18 of the 19 plasmids, one end of the deletion was localized in M13 between the *Sau*96.1 site at nucleotide 5724 and the *Hin*fI site at nucleotide 5788. The other end of the deletions was localized at different positions within pHV33. Two clusters were, however, observed, the first between nucleotides 1620 and 1720 of pBR322 (five deletions, labeled pHV671Δ1 to -Δ5), and the second between nucleotides 1350 and 1450 of pC194 (four deletions, pHV672Δ12 to -Δ15). An additional deletion was detected in one of the plasmids (pHV672Δ18). The remaining plasmid was generated by a deletion ending upstream of M13 nucleotide 5120. This shows that 90% of deletions (18/20) ended in

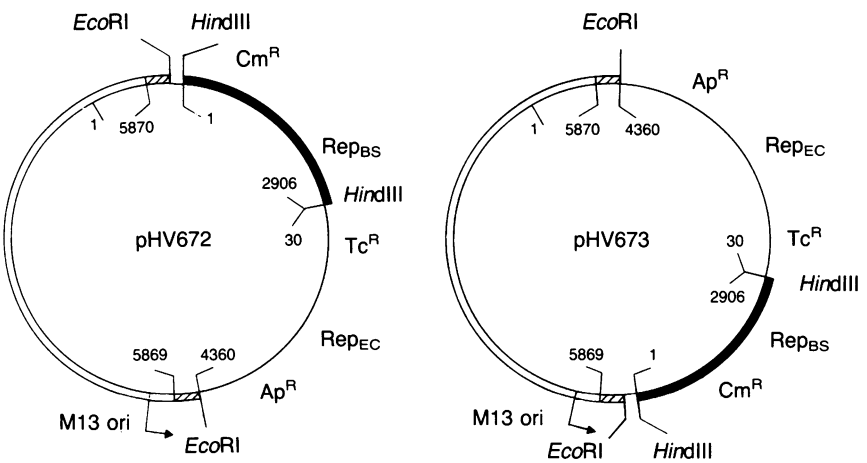

FIG. 1. Schematic representations of pHV672 and pHV673. Thin lines represent pBR322 sequences; thick lines, pC194 sequences; double lines, M13 sequences; hatched regions, *lac* sequences. Only the restriction sites used for cloning are shown. Rep_{EC}, Rep_{BS}, and M13 ori denote replication regions of pBR322, pC194, and M13, respectively. Arrow indicates the M13 origin and the direction of viral replication. Numbers indicate nucleotides coordinates of pC194, pBR322, and M13. Genes conferring resistance to ampicillin, chloramphenicol, or tetracycline are indicated by the phenotype symbols Ap^R, Cm^R, and Tc^R, respectively.

a 63-bp region of M13. A similar result was obtained for six plasmids derived from pHV673. Five of these were generated by deletions ending between nucleotides 5724 and 5787 of M13. The remaining plasmid was generated by a deletion ending within the *lac* sequences carried on M13 mp2. This shows that >80% of deletions occurring within pHV673 have one end in the same 63-bp M13 region.

Sequence Analysis. The deletion endpoints were sequenced in 10 plasmids, of which 7 were derived from pHV672 and 3 from pHV673 (Fig. 2). In three cases where no homology was found at the junctions, the last M13 nucleotide retained was nucleotide 5781, which is adjacent to the nick introduced into the phage replication origin by the gene II protein (12, 35). Homologies from 2 to 6 bp long between the recombining sequences were detected by an analysis of the remaining seven plasmids. An unambiguous localization of the site of deletion was therefore not possible, but the nucleotide 5781 could have been involved in all cases.

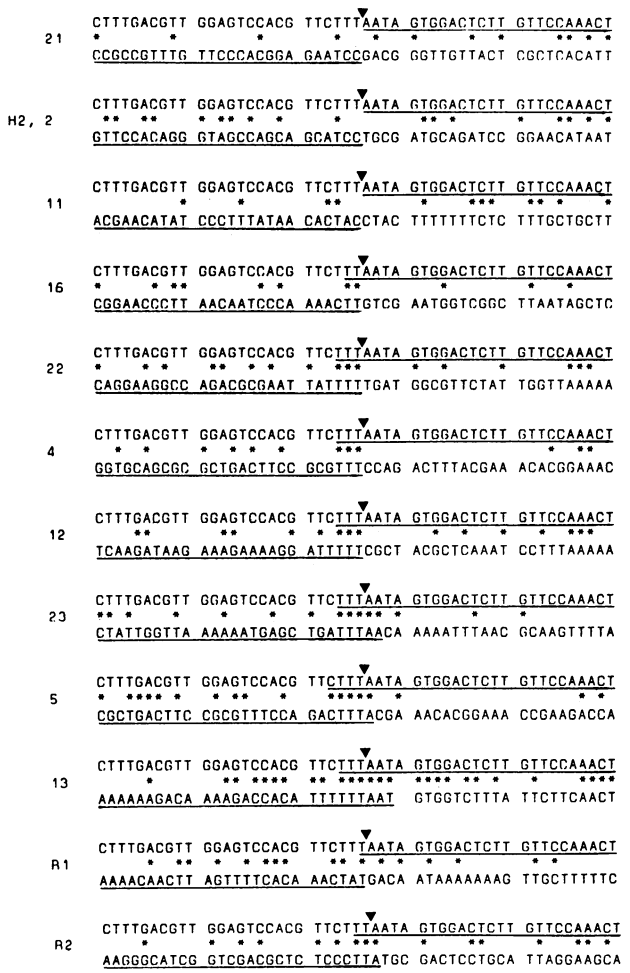

FIG. 2. DNA sequences involved in the recombination events between the M13 replication origin and other pHV672 or pHV673 regions. All sequences are shown in the 5'→3' direction. The top sequence of each pair is that of the M13 origin (between nucleotides 5755 and 5804), and the bottom sequence, that of the recombining plasmid region. Asterisks indicate identical nucleotides. The underlined sequence was determined by analyzing deletion plasmids indicated by numbers. In each sequence the solid triangle indicates the position of the nick. For each plasmid, the site of recombination with the origin region was as follows: Δ2, 1681 of pBR322; Δ4, 1631–1633 of pBR322; Δ5, 1621–1624 of pBR322; Δ11, 2029 of pC194; Δ12, 1421–1423 of pC194; Δ13, 1372–1378 of pC194; Δ16, 333–334 of pC194; Δ21, 707 of *lac*; Δ22, 777–779 of *lac*; Δ23, 5893–5898 of M13; ΔR1, 1611 of pC194; ΔR2, 640 of pBR322. The first 10 plasmids, isolated from the Rep$^+$ *E. coli*, are ordered by increasing homology at the deletion endpoints.

Transduction Analysis. Zinder and coworkers (35) have shown that plasmids containing an intact M13 replication region are transduced very efficiently by M13, those that lack nucleotides 5771–5776 are transduced about 1% as efficiently, and those that lack nucleotide 5777, 0.001% as efficiently. Eight of the sequenced plasmids deleted at nucleotide 5781 were transduced 0.01% as efficiently as the control plasmids pHV698 and pHV702, which carried an entire M13 replication origin. Plasmids pHV672Δ5, which had the correct M13 sequence starting from nucleotide 5776, and pHV672Δ13, which had an almost correct M13 sequence starting from nucleotide 5771, were transduced 1% as efficiently. The 13 other plasmids derived from pHV672 and pHV673, which were studied by restriction only, were transduced poorly (10 at a frequency of 10^{-4}, 3 at a frequency of 10^{-2}). This suggests that they were also generated by a deletion starting at the nick in the M13 replication origin.

Inactivation of the Gene II Protein. To determine whether the nucleotide neighboring the nick recombines frequently because of particular flanking sequences, or because of the gene II protein activity, we decided to inactivate gene II and examine the deletions formed in the absence of the nicking protein. For that purpose, a *Hin*dIII linker 8 bp long was inserted in the *Hpa* I site of pHV672. This introduced a frameshift mutation in the M13 gene II, which should allow synthesis of a polypeptide 150 amino acids long, instead of the gene II protein, which is 410 amino acids long. Two independent isolates, pHV672H1 and pHV672H2, were used to transform *E. coli* competent cells. Both transformed about 1% as efficiently as pHV672. All the transformants obtained were resistant to ampicillin, tetracycline, and chloramphenicol (Table 1). If the recipient cells were previously infected with the M13 mp2 phage, which codes for the wild-type gene II protein, the two mutant plasmids transformed only about 80–85% less efficiently than pHV672. (*recA recB* host was used in this experiment to prevent recombination between the transforming plasmid and the resident phage. These mutations do not affect formation of viable plasmids; see below.) Two types of transformants, Ap^R and Ap^R Tc^R, were obtained in similar proportions. These results indicate that deletions occur more frequently and are of a different type in the presence of the gene II protein, provided *in cis* (as in pHV672) or *in trans*.

Deletion endpoints were mapped in four plasmids isolated in an *E. coli* host that did not contain M13 mp2; two of these plasmids were derived from pHV672H1 and two from pHV672H2. All four plasmids carried the *Sau*96.1 site at nucleotide 5724, which shows that they resulted from deletions ending at least 56 bp from the nick. As expected, these plasmids were transduced by M13 mp2 as efficiently as the control plasmid pHV702, which carries an intact M13 replication region. Three plasmids were isolated from *recA recB E. coli* cells harboring M13 mp2; two of these plasmids were derived from pHV672H1 and one from pHV672H2. They were analyzed as described above. All three lacked the *Sau*96.1 site at the nucleotide 5724, carried the *Hin*fI site at the nucleotide 5788, and were transduced 10^4 times less efficiently than the control plasmid. One of them was sequenced and shown to be identical to pHV672Δ2. Deletions are therefore generated from nucleotide 5781 only in the presence of the functional gene II protein, supplied either *in cis* or *in trans*.

***E. coli* Mutants.** pHV672 and pHV673 were used to transform Rep$^-$ cells (strain D162), in which M13 phage cannot replicate (14). The relative transforming efficiency (compared to that of pHV33), the phenotypes of transformants, and the sizes of plasmids harbored by the transformants were similar to those observed with the Rep$^+$ strain GY2098.

Five plasmids obtained from pHV672 were subjected to restriction analysis. One plasmid was generated by a deletion ending upstream of the origin of replication of M13. In the four other cases, one end of the deletion was localized between nucleotides 5724 and 5787 of M13, while the other end was between nucleotides 1630 and 1680 in pBR322 for the largest deletion and between nucleotides 1360 and 1420 in pC194 for the smallest. The deletions started at or in the immediate vicinity of nucleotide 5781 in the two plasmids pHV672ΔR1 and pHV672ΔR2, as determined by sequence analysis (Fig. 2). Deletion thus appears to be independent of phage replication.

pHV672 and pHV673 were used to transform the following set of isogenic strains, all rendered *hsdR*: AB1157 (Rec^+), AB2470 (*recB*), JC5787 (*recC*), JC5519 (*recBC*), JC5495 (*recA recB*), JC5547 (*recA recB recC*), JC11445 (*sbcA*), JC11451 (*sbcB*), JC8679 (*recB recC sbcA*), JC8471 (*recL*), and JC9239 (*recF*). There were no significant differences in (*i*) transforming efficiencies, which varied between 0.3 and 3 for pHV672 and 0.5 and 1.5 for pHV673, relative to the transforming efficiencies with the Rec^+ strain, or (*ii*) phenotypes of the resulting clones. Deletions therefore appear to be independent of the *E. coli* recombination functions we examined.

DISCUSSION

To investigate illegitimate recombination in *E. coli*, we decided to isolate plasmid genomes having undergone deletions and to determine their structure. Isolation of such genomes was facilitated by the use of plasmids viable in *B. subtilis* but not in *E. coli* unless they lose some of their sequences by deletion. The reasons for the nonviability of pHV672 and pHV673 in *E. coli* are not clear. The two plasmids are composed of three replicons, M13 mp2, pBR322, and pC194. Our failure to construct hybrids between M13 mp2 and pBR322 (unpublished data) suggests that these may already be nonviable. Hybrids between R199 (a cloning vector derived from the filamentous phage f1) and pBR322 are, however, viable (21). The two phage cloning vectors are different, since M13 mp2 carries a 789-bp insert within a domain necessary for replication (36) and encodes a mutated gene II protein that compensates for this defect (37), whereas R199 carries a 4-bp insert within a domain not necessary for replication and encodes a wild-type gene II protein (21, 38). It is not known whether and how these differences affect hybrid viability. Notwithstanding our ignorance of the reasons for the nonviability of plasmids used in this work, they proved to be a very efficient tool for studying deletions.

Eighty to ninety percent of deletions in pHV672 and pHV673 were generated by joining the 5′ phosphate adjacent to the nick introduced in the M13 replication origin by the gene II protein (12, 35) and a nucleotide located elsewhere in the plasmid genome. This process may be a novel type of illegitimate recombination. Three classes of breakage–reunion models may be proposed to describe it.

(*i*) Gene II protein could have an illegitimate recombinase activity. This protein initiates phage replication by nicking replicative form I (RFI) to generate a 3′-hydroxyl end, which serves as a primer for DNA synthesis, and a 5′-phosphoryl end. It later also terminates phage replication by cleaving the nascent single strand from the replication intermediate at the end of a round of replication and sealing it to form a covalently closed circular molecule (39, 40). The cleavage step of the termination reaction is thought to produce a single-stranded DNA molecule with the gene II protein attached at the 3′-hydroxyl end (39). This end is joined during the sealing step to the 5′-phosphoryl end of the molecule in a reaction reminiscent of those described for topoisomerases I (7, 8). If the protein sometimes cleaves at sites other than that normally used for termination and then performs the sealing reaction with the 5′-phosphoryl end resulting from the nicking at the correct initiation site, it could generate molecules such as those observed in this study. The termination signal is complex: it encompasses the gene II protein recognition sequence and the palindromic region extending for 12 nucleotides to the 5′ side of the nicking site (35), and arguments against model *i* may be based on the absence of homology between this complex signal and the sequence at the site of recombination. If correct, however, this model would lend support to the speculation that the nicking–closing enzymes are involved in illegitimate recombination (11). It may be possible to test this hypothesis with *in vitro* experiments using the purified gene II protein.

(*ii*) The nick could be recombinogenic. Nucleotides adjacent to the nick appear to be able to recombine illegitimately. This has been shown in this study for the 5′ phosphate and has been observed previously for the 3′ hydroxyl adjacent to the nick in the filamentous phage replication origin (40, 41). Illegitimate recombination could occur only at nicks in replication origins (see below) or more generally at nicks (and possibly other lesions such as gaps) anywhere in DNA molecules. The simplest model illustrating the latter hypothesis is that a DNA ligase-like activity can join a 5′-phosphate nucleotide to a 3′-hydroxyl nucleotide located elsewhere in the genome. T4 ligase was shown to be able to join nucleotides separated by a gap *in vitro* (42). Its flush-end joining activity (43, 44) illustrates the fact that sequence homology is not necessary for ligation. Experiments with ligase mutants may allow further testing of this model.

(*iii*) A nick in the replication origin may be recombinogenic. The M13 replication origin consists of two domains, the essential domain (A), extending for 12 nucleotides on the 5′ side of the nick and 28 nucleotides on the 3′ side, and the dispensable domain (B), extending for a further 100 nucleotides on the 3′ side (36). The latter has been suggested as the site of formation of the replication fork (37), and it is conceivable that the nick is converted into a recombinogenic entity during this process. This may be visualized as a melting of the double-stranded DNA in the region of the nick, which would generate a putatively recombinogenic single DNA strand. Experiments with Rep^- cells, in which M13 does not replicate (14), suggest that extensive movement of the fork is not needed for recombination at the nick. This model predicts that nicks in other replication origins would also recombine illegitimately. Since the *oriV* of the F plasmid recombines illegitimately (ref. 45; see also below), it would be interesting to determine whether the vegetative replication of the plasmid F is initiated at a nick, as is the transfer replication (46).

The 5′-phosphate nucleotide adjacent to the nick recombined at many sites in pHV672 and pHV673, but some of these sites were clustered. Two such clusters were detected in pHV672 by restriction mapping, one in the region between nucleotides 1620 and 1720 of pBR322, and another in the region between nucleotides 1350 and 1450 of pC194 (pHV672Δ1 to -Δ5 and -Δ12 to -Δ15, respectively). Four recombination events from the first cluster and two from the second have been sequenced. They occurred within a 60-bp-long pBR322 region (nucleotides 1620–1680, pHV672Δ2, -Δ4, -Δ5, and -ΔH2, Fig. 3) and a 50-bp-long pC194 region (nucleotides 1372–1423, pHV672Δ12 and -Δ13, Fig. 3). No conspicuous sequence homology between these regions and the M13 origin was found except in the case of pHV672Δ13. In each of these regions, a palindromic sequence exists (Fig. 3). It is interesting that the replication origin, *oriV*, of the plasmid F recombined within the same pBR322 region (48). The major site for this recombination coincided with that identified in plasmid pHV672Δ5 (Fig. 3), except that the opposite pBR322 strand was used. Another site, only 2 bp away, was present on the pBR322 strand identical with that which recombined in our experiments (48). It is conceivable

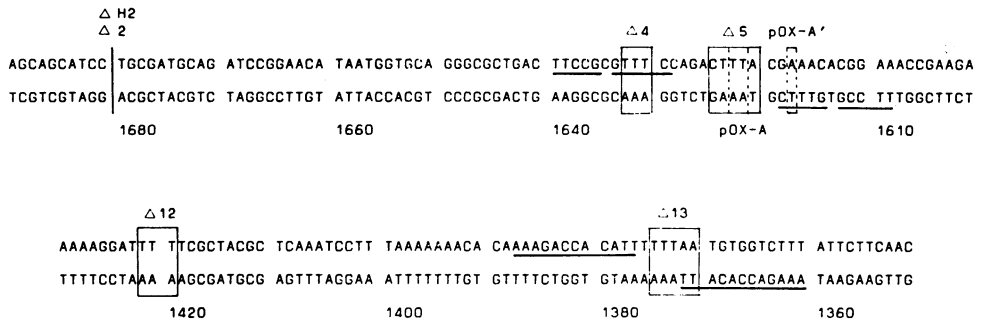

FIG. 3. The nucleotide sequences of pBR322 (nucleotides 1690–1601, *a*) and pC194 (nucleotides 1431–1351, *b*). The sites of recombination with M13 and pOX38 (47) are indicated by solid or dashed boxes, respectively. Δ followed by a number designates a plasmid generated from pHV672; pOX-A and pOX-A' designates those generated from pOX38 (48). Palindromic sequences are underlined.

that a structure particularly prone to recombine with the replication origins may be generated by the palindromic sequences. Computer analysis of the pBR322 and pC194 sequences has shown, however, the existence of a number of other palindromes that did not recombine with the nick. Unlike the above "hot" regions, the ampicillin-resistance gene of pBR322 appeared particularly refractory to recombination, as judged by the fact that no Ap^S clones were observed among the Tc^R transformants obtained with pHV673 DNA (Table 1 and Fig. 1). The frequent involvement of certain plasmid regions and not of others in recombination with the nick may explain, at least in part, the finding that viable deletions were isolated more frequently in pHV672 than in pHV673 (Table 1). Recombination between the nick and the hot regions in pBR322 and pC194 would generate viable genomes, carrying the pBR322 replication origin, in the case of pHV672 but not in the case of pHV673 (Fig. 1).

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