A New Type of Illegitimate Recombination Is Dependent on Restriction and Homologous Interaction

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Illegitimate (nonhomologous) recombination requires little or no sequence homology between recombining DNAs and has been regarded as being a process distinct from homologous recombination, which requires a long stretch of homology between recombining DNAs. Under special conditions in *Escherichia coli*, we have found a new type of illegitimate recombination that requires an interaction between homologous DNA sequences. It was detected when a plasmid that carried 2-kb-long inverted repeats was subjected to type II restriction in vitro and type I (*Eco*KI) restriction in vivo within a Δ *rac* *recBC* *recG* *ruvC* strain. Removal of one of the repeats or its replacement with heterologous DNA resulted in a reduction in the level of recombination. The recombining sites themselves shared, at most, a few base pairs of homology. Many of the recombination events joined a site in one of the repeats with a site in another repeat. In two of the products, one of the recombining sites was at the end of one of the repeats. Removal of one of the *Eco*KI sites resulted in decreased recombination. We discuss the possibility that some structure made by homologous interaction between the long repeats is used by the *Eco*KI restriction enzyme to promote illegitimate recombination. The possible roles and consequences of this type of homologous interaction are discussed.

Illegitimate recombination of DNA is defined as recombination between sequences containing little or no base pair sequence homology (34). The term is limited here to recombination processes taking place at low frequencies at numerous sites on the chromosome, as opposed to recombinational events at specific sites such as within immunoglobulin genes. The possible underlying mechanisms that have been proposed include the joining of double-stranded ends after degradation (47) and strand slippage during replication (1).

Illegitimate recombination has been regarded as a process distinct from homologous recombination, which involves much longer regions of homology (typically in the kilobase range) (11) and depends on a different set of gene products (34). However, it has been suggested that some types of DNA lesions, such as double-strand DNA breaks, can trigger both illegitimate and legitimate (homologous) recombination (3).

Analysis of aberrant homologous recombination products in mammalian cells has led to the proposal that interaction between two homologous DNA regions results in illegitimate recombination of one of the interacting sites with a third, uninvolved site (9, 22, 48). One of the possible molecular mechanisms that assumes the existence of a Holliday intermediate is illustrated in Fig. 1. According to this model, illegitimate recombination is a consequence of the destruction of an intermediate involved in homologous recombination, with some illegitimate recombination events possibly taking place at the ends of long regions of homology shared between the recombining DNAs. These illegitimate events have indeed been observed (9, 48).

This model was able to explain several of the aberrant features of mammalian homologous recombination (9, 48) as well as genome rearrangements (22). Also, it led to the assumption

of a random walk of the connecting point along the homology and destruction of the connecting point at the ends of the homology. This formulation permitted quantitative explanations of the relationship between the frequency of homologous recombination and the length of sequence homology in various organisms (10, 11).

In this work, we further pursue the analysis of this homology-associated but nonhomologous recombination event in the prokaryote *Escherichia coli*. We have exposed a plasmid carrying kilobase-long inverted repeats to restriction in vitro and in vivo. Illegitimate recombination detected at a high frequency under these conditions was dependent on the presence of sequence homology.

MATERIALS AND METHODS

Bacterial strains. DH10B (*recA1* Δ *rac* r_K^- m_K^-) (14) was from our laboratory collection. JC5519 (*recB21* *recC22* Δ *rac*) (61) was a gift from Takeshi Kato. JC8679 (*recB21* *recC22* *rac*⁺ *sbcA23* r_K^+ m_K^+) (12) and JC8691 (*recB21* *recC22* *rac*⁺ *sbcA23* *recE159* r_K^+ m_K^+) (12) were gifts from John Clark. The *Ral*⁺ (restriction alleviation) phenotype conferred by the *lar* gene on the *Rac* prophage (20) was checked by spotting *Eco*KI-modified and *Eco*KI-unmodified lambda *vir* on a bacterial lawn. BIK1489 (*recB21* *recC22* *rac*⁺ *sbcA23* *recG258::Tn10mini-kan* *ruvC53* *eda51::Tn10* r_K^+ m_K^+) is a *ruvC53* *eda51::Tn10* derivative of N2796 (a *recG258::Tn10mini-kan* derivative of JC8679 from Robert Lloyd [36]), made by transduction with P1 from BIK1050, a *ruvC53* *eda51::Tn10* derivative of JC8679 (33). BIK2589 and BIK2590 were made by the same procedure. BIK1490 (*recB21* *recC22* Δ *rac* *recG258::Tn10mini-kan* *ruvC53* *eda51::Tn10* r_K^+ m_K^+) was isolated as a spontaneous fast-growing variant from BIK1489. BIK1490 retains *recB21*/*recC22*, *recG258*, and *ruvC53* mutations because it can donate these mutations in P1 transduction. BIK2591 was isolated from BIK2590 by the same procedure. Its *recB21* *recC22* allele, *recG258::Tn10mini-kan* allele, and *ruvC53* *eda51::Tn10* allele were restored to wild-type alleles by a series of backcrosses to give strain BIK2592.

Plasmids. Plasmid pIK43 is described in the legend to Fig. 2A. pIK32 lacks the lower *neo* copy of pIK43 and was described previously (48). pIK151, a derivative of pIK43, has a deletion from the *Eco*RV site to the *Kpn*I site that removes an *Eco*KI site (Fig. 2A). pIK154 was made by replacing the lower *neo* segment of pIK43 (Fig. 2A) with a 1,243-bp-long fragment containing the chloramphenicol resistance gene (*cml*). This fragment is a *Sal*I-*Bam*HI fragment from pBR322::*cml*, which was made by inserting a 777-bp-long *Bam*HI-*Bam*HI *cml* cartridge (6) into the *Bam*HI site of pBR322. Two plasmids, pJC1509 (5) and pRAC3 (62), were generous gifts from John Clark.

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FIG. 1. A possible mechanism for homology-associated non-homologous recombination. Two DNAs with homology in one region (A) recognize each other in a process of homologous pairing and form a cross-stranded (Holliday) structure (B). The branch point moves along the homologous region in a process known as branch migration (C). The branch eventually encounters the end of the homology (C). Some aberrant structure such as a double-strand break is formed at the branch point when the branch point is within the homology (D) or at the end of the homology (E). This structure leads to nonhomologous recombination of a site at the branch point with a third, unrelated DNA (F and G).

Bacteriophages. P1 *vir* and lambda *vir* were from our laboratory collection. Kohara's ordered lambda clone 263 (24) was from Akira Ishihama.

Transformation. The methods used for transformation have been previously described in detail (32, 54, 64). The parental plasmids pIK43, pIK32, pIK151, and pIK154 were prepared from DH10B (*recA1* Δ *rac* r_K^- m_K^-) and were purified by banding in cesium chloride-ethidium bromide gradients. They were cut thoroughly with *Xho*I. The cut and uncut plasmids were used to transform various *E. coli* strains by electroporation in a Gene Pulser (Bio-Rad). At the end of transformation, aliquots of the transformation mixture that had been incubated at 37°C for 60 min were immediately spread on ampicillin agar plates (ampicillin [50 μ g/ml] with methicillin [200 μ g/ml]) directly or after dilution. Methicillin was added to inhibit the generation of satellite colonies around a true ampicillin-resistant (*Amp*^r) colony. Plasmid DNA was isolated from these transformants by the alkaline method (49) and analyzed with restriction enzymes (63).

Southern hybridization and sequence analysis. Southern hybridization was carried out as described previously (31). Parts of the product plasmids were subcloned into pUC118 and/or pUC119 (59). DNA sequences were determined with T7 DNA polymerase, using the M13 universal primer (5' CGACGTTGTA AAACGACGGCCAGT) or the M13 reverse primer (5' CAGGAAACAGC TATGAC) in a Pharmacia ALF DNA sequencer as described previously (48). The sequences were analyzed with Genetics Computer Group programs. STEM-LOOP was used in the search for palindromes around each of the recombination sites. Minimum stem, maximum loop size, and minimum bonds/stem were set to 6, 20, and 12.00, respectively.

RESULTS

Double-strand break repair in the presence of *Eco*KI restriction. We first noticed the presence of nonhomologous recombination dependent on homologous sequences when we modified our system for the analysis of homology-dependent double-strand break repair (23, 32, 33, 54, 64).

Our substrate plasmid (pIK43 [Fig. 2A]) carries, in inverted orientations, two copies of a 2-kb-long segment containing the *neo* gene. The lower and upper segments have a deletion that removes a different region at the end of the *neo* gene. Because

an oligonucleotide containing a site for the restriction enzyme *Xho*I is inserted at the deletion site on the upper segment, cleavage with *Xho*I generates a long double-stranded gap corresponding to the size of the deletion (approximately 300 bp long) compared with the lower sequence.

We introduced this gapped plasmid [Fig. 2B(i)] into *E. coli* cells, and immediately after the time allowed for the expression of antibiotic resistance, the cells were spread on agar plates containing antibiotics. Repair of the double-stranded gap through gene conversion by using the corresponding wild-type sequence in the lower segment as a template (Fig. 2A) resulted in two types of products as shown in Fig. 2B(ii) and (iii). The overall transformation efficiency as measured by counting the number of *Amp*^r colonies was not affected very much by the double-strand break. This double-strand break repair was detected in *E. coli* JC8679 (*recBC sbcA*) but not among some other strains such as AB1157 (*rec*⁺) or DH5 (*recA*) (58).

In these early experiments, the two *Eco*KI sites of the plasmid (Fig. 2A) were supposedly modified by methylation, as the plasmid molecules were prepared in bacterial strains DH1 and DH5 (15), carrying *Eco*KI modification function. In the present work, we prepared this plasmid in a strain (DH10B) lacking the *Eco*KI modification (see Materials and Methods) so that the *Eco*KI sites remained unmodified. The plasmid was then cut with *Xho*I in order to create a double-strand break. Restriction was efficient in the sense that it reduced the transformation efficiency nearly 1,000-fold in a recipient strain that was recombination deficient and lacked the *Eco*KI restriction system (DH10B [*recA1* Δ *rac* r_K^- m_K^-]) (Fig. 3B, last row). In JC8679 (*recB21 recC22 rac*⁺ *sbcA23 r_K*⁺ m_K ⁺), we found the decrease in the transformation efficiency after *Xho*I cleavage to

FIG. 2. Detection of homology-associated nonhomologous recombination. (A) Substrate plasmid pIK43. The two homologous segments including the *neo* gene are drawn as parallel lines. The top *neo* segment has a 283-bp-long deletion (deletion a) between the two *NaeI* sites. The *NaeI* site was inactivated by insertion of an *XhoI* linker sequence, 5' CCTCGAGG. The bottom *neo* segment has a 248-bp-long deletion (deletion b). The two deletions are separated by a 506-bp region of homology and are flanked by 1,079 bp of homology to the immediate left and 231 bp of homology to the immediate right. The leftward, unique segment (2,321 bp), including the *amp* (ampicillin resistance) gene and the replication origin, is derived from pBR322. The rightward, unique segment (8,297 bp) is derived from simian virus 40 and bovine papillomavirus type 1. The entire plasmid length is 14,795 bp. The restriction enzymes and their site coordinates are as follows: *EcoRI* (RI), 8024 and 14793; *XhoI* (X), 1082; *HindIII* (HIII), 2070 and 4924; *KpnI*, 9370; *EcoRV* (RV), 10367; *NaeI* (N), 11109, 11392, 12590, and 12750; *SalI*, 12472; *EcoKI*(1), 14458 (clockwise); *EcoKI*(2), 10119 (counterclockwise). The construction and sequence of the plasmid were described previously (14). This plasmid was prepared from DH10B (*recA1 Δrac rK⁻ mK⁻*). (B) Experiments. Cutting pIK43 with *XhoI* (arrow) produces a double-stranded gap of 283 bp (compared with the lower segment) in the upper segment (i). The gapped substrate was transferred into *E. coli* JC8679 (*recB21 recC22 rac⁺ sbcA23 rK⁺ mK⁺*) by electroporation. The cells that reconstituted a circular plasmid were selected on ampicillin agar plates. In these clones there were plasmids in which the double-stranded gap had been repaired by copying the homologous sequence of the lower segment. The repair took place with (iii) or without (ii) crossing-over of the flanking sequences. On the other hand, when the gapped substrate was transferred into *E. coli* BIK1490 (*recB21 recC22 Δrac recG258 ruvC53 rK⁺ mK⁺*), most of the clones had a rearranged plasmid [(iv) and (v)]. Some of them turned out to carry a nonhomologous junction at the end of the homologous region [(iv)].

be much smaller (Fig. 3B, top row). The decrease was comparable to that found with the *EcoKI*-modified preparations and suggested the occurrence of double-strand break repair. In a *recE* mutant derivative of this strain, JC8691 (*recB21 recC22 rac⁺ sbcA23 recE159 rK⁺ mK⁺*), this level was decreased to the level of the negative control (DH10B [*recA1 Δrac rK⁻ mK⁻*]) (Fig. 3B, second and last rows). This result indicates that the double-strand break repair is dependent on *recE* function as in the absence of restriction (33, 55). Results essentially the same

as those described above were obtained with a derivative of JC8679 carrying *recG* and *ruvC* mutations (BIK1489 [*recBC sbcA recG ruvC rK⁺ mK⁺*]) (Fig. 3B, third row). Therefore, the double-strand break repair takes place in the absence of a functional *RecG* protein, branch migrase (37), and *RuvC* resolvase (7, 18).

Most of the transformants carried a plasmid of the same size as the parental plasmid (Fig. 4A). In most of the products, the double-strand break had been repaired through gene conversion with the homologous duplex (Fig. 3C, first row) as judged from *XhoI* and *EcoRI* restriction analysis (Fig. 2A). The remaining products belonged to the parental type. They presumably represent descendants of circular plasmid molecules that had escaped cutting with *XhoI* in vitro or that had formed by rejoining of the *XhoI*-cleaved ends in vivo after transfer to the cells.

Thus, the homology-dependent double-strand break repair took place in spite of the presence of the *EcoKI* restriction system in these strains. This finding turned out to be in harmony with the recent discovery of a gene (*lar*) on *Rac* prophage that inhibits *EcoKI* restriction (20).

Excision of *Rac* prophage from *rac⁺ sbcA recBC recG ruvC* strains. The above-described *recBC sbcA ruvC recG* strain grew slowly and made very small colonies. They spontaneously gave rise to faster-growing variants, which were readily identifiable as larger colonies. When we constructed two more *recBC sbcA ruvC recG* strains, plating of the cells resulted once again in the appearance of fast growers. These fast growers were defective in conjugative recombination (data not shown).

We verified, through backcrosses, that these fast-growing variants had retained the *recBC*, *ruvC*, and *recG* mutations. Thus, reversion of these mutations cannot be responsible for the fast-growth phenotype. The restriction alleviation phenotype of the parental strain conferred by the *lar* gene on the *Rac* prophage (Fig. 5) (20) was found to be lost in the fast growers (data not shown). This result suggested that some type of mutational alteration had taken place on the *Rac* prophage in these derivatives.

We analyzed the *Rac* prophage regions (Fig. 5A) of these fast growers and their descendants by Southern hybridization. Our probe pRAC3 (62) spans the left attachment site of the *Rac* prophage (Fig. 5A). With *EcoRI*, the internal 2.8-kb band was found to be missing (Fig. 5). The 8.2-kb fragment, which covers the left attachment site, was also missing and was replaced by a 10-kb band. This is approximately the same size as the fragment carrying the bacterial attachment site without the *Rac* prophage. With *EcoRI* and *HindIII*, the expected 2.4-, 2.5-, and 2.8-kb fragments were also found to be missing. Instead, a 4.2-kb band corresponding to the expected size of the bacterial attachment site appeared. These results are exactly those that one would expect if the *Rac* prophage had been precisely deleted. The excision was confirmed by two more probes: the pJC1509 probe within the *Rac* prophage (4) and lambda clone 263 of Kohara's ordered *E. coli* genome libraries (24) (data not shown).

The parental strain (*recBC sbcA ruvC recG*) showed small amounts of DNA corresponding to the excision (Fig. 5B and data not shown). Excision of *Rac* prophage appears to have taken place in a small fraction of the cell population during growth, consistent with the hypothesis that excision itself is responsible for the fast-growth phenotype of these descendants. Further experiments are necessary to examine the interesting possibility that *recE* and *recT* genes on *Rac* prophage generate DNA forms that are toxic unless processed either by *RecBC*, *RuvC*, or *RecG*.

FIG. 3. Gene conversion versus nonhomologous rearrangements. (A) Strains and genotypes. (B) Transformation efficiency. A fixed amount of cells from each of the indicated strains was transformed by electroporation with 0.5 μ g of pIK43 (Fig. 2A) DNA unmodified with *Eco*KI methylase (see Materials and Methods), either intact or after having been cut with *Xho*I. Aliquots of the transformation mixture were spread on ampicillin agar plates and then incubated overnight. The number of *Amp^r* transformants from cut DNA relative to that from intact DNA was plotted along the horizontal axis. The transformation efficiencies in these strains are as follows (*Amp^r* transformants from 0.5 μ g of cut DNA/*Amp^r* transformants from 0.5 μ g of uncut DNA): JC8679, $1.88 \times 10^4/6.1 \times 10^5$ and $2.2 \times 10^4/5.1 \times 10^5$; JC8691, $2.5 \times 10^4/2.4 \times 10^4$; BIK1489, $6.5 \times 10^4/4.9 \times 10^5$ and $2.1 \times 10^5/5.8 \times 10^5$; BIK1490, $1.50 \times 10^4/1.41 \times 10^5$ and $1.75 \times 10^5/5.7 \times 10^5$; DH10B, $9.1 \times 10^2/4.8 \times 10^5$ and $1.13 \times 10^5/9.4 \times 10^7$. (C) Classification of the products. Plasmid DNA was recovered from the *Amp^r* colonies that had been transformed with cut DNA and was classified into gene conversion groups and nonhomologous recombination groups by digestion with *Xho*I and *Eco*RI. n, number of transformant colonies analyzed.

Detection of nonhomologous recombination and its dependence on the presence of homology. In the fast-growing derivatives, including BIK1490 (*recB21 recC22 Δrac recG258 ruvC53 rK⁺ mK⁺*) (Materials and Methods), the double-strand break repair of unmodified or modified gapped plasmid (pIK43) was not detected (Fig. 3, fourth row and data not shown). This was as expected because double-strand break repair with this substrate is dependent on the *Rac* prophage (33, 55).

The effect of *Xho*I cleavage in vitro on the transformation efficiency of the *Eco*KI-unmodified plasmid in this strain was not very different from that in the parental strain (Fig. 3B, third and fourth rows). The products were, however, quite different (Fig. 3C, third and fourth rows). Most of them had different sizes (Fig. 4B) and turned out to have a form that could not be explained by homologous recombination (Fig. 3C, fourth row), as described in detail below. The remainder were the same as the parental type, and none of the plasmids examined could be classified as resulting from gene conversion.

Our substrate plasmid carries two copies of the *neo* segment; the first copy was cut with *Xho*I, and the second remained intact (Fig. 2A). When a plasmid lacking the second *neo* copy (pIK32) was used, we found a lower relative transformation efficiency that was comparable to the level of the negative control (DH10B [*recA1 Δrac rK⁻ mK⁻*]) (Fig. 6A). A similar

decrease was observed when the second *neo* copy (2,099 bp) was replaced by a heterologous DNA segment of comparable size (1,243 bp) carrying the *cml* gene (Fig. 6B). Thus, this form of recombination apparently requires the presence of a second homologous copy of the gene.

When BIK1490 (*recB21 recC22 Δrac recG258 ruvC53 rK⁺ mK⁺*) was transformed with pIK43, unmodified in vivo by *Eco*KI, and uncut in vitro by *Xho*I, only 1 of the 32 *Amp^r* transformant clones was of the rearrangement type; the remaining (31) were of the parental type.

Structures of the products. The structures of the rearranged products in the foregoing experiment with pIK43 were analyzed with the restriction enzymes *Xho*I, *Eco*RI, *Hind*III, *Eco*RV, and *Sal*I (Fig. 2A). All of the products examined can be interpreted to be a result of one recombination event that joined two sites along the plasmid. Figure 7B summarizes the classification of the products with respect to the boundary of the two *neo* segments (Fig. 7A). All of them lack the *Xho*I site that had been cleaved before transfer. Many of them joined one site in the top *neo* segment with another site in the bottom *neo* segment (I-III). The majority of the products carried at least one nonhomologous recombination site in the *neo* segment (all except IV-II and IV-IV). Only a few products joined two sites that flank the *Xho*I break site (I-I). These results are

FIG. 4. Plasmids in the transformants. (A) Gene conversion-type products from the transformants of JC8679 (*recB recC rac⁺ sbcA rK⁺ mK⁺*); (B) plasmids from the transformants of BIK1490 (*recB recC Δrac recG ruvC rK⁺ mK⁺*). Ten belong to the nonhomologous rearrangement type, and two belong to the parental type. Marker lanes contain a circular ladder (Bio-Rad), which consists of pairs of a supercoil and an open circle.

FIG. 5. Chromosomal Rac prophage region of a *recBC sbcA recG ruvC* strain of *E. coli* and its fast-growing derivative. (A) The map is based on previous data (5, 20). Rac prophage is indicated by a thick line. Approximate locations of its *lar*, *recT*, and *recE* genes are indicated. E, *EcoRI* site; H, *HindIII* site; attL, left attachment site; attB, bacterial attachment site without Rac prophage. The DNA fragments expected after digestion with the restriction enzymes are shown. pRAC3 is a probe. (B) Southern hybridization. The probe is pRAC3. First, fourth, and seventh lanes, *recBC sbcA recG ruvC* strain BIK2590 (see Materials and Methods); second, fifth, and eighth lanes, a descendant of a fast-growing derivative of this strain (BIK2592) (see Materials and Methods); third, sixth, and ninth lanes, a strain without Rac prophage (JC5519). The numbers indicate fragment lengths in kilobases.

consistent with the previous data and are in harmony with the hypothesis that homologous interaction between the two *neo* segments was responsible for most of the nonhomologous recombination events.

The DNA sequences at the junctions of the recombinant sites were determined for some of the products (Fig. 8). At most only 1 to 4 bp of homology was detected between the recombining sites of the DNA sequences. This feature is often found in illegitimate recombination in *E. coli* (1). There were no DNA sequences similar to those that have been implicated in illegitimate recombination in *E. coli*, such as large palindromes, integration host factor-binding sites (5' C/TAANN NNTTGATA/T), or Fis-binding sites (5' GNNYRNNTNNY RNNC) (5, 10, 14), within a distance of 50 bp of either of the recombining sites in any of these products (see Materials and Methods).

Two of the products had one nonhomologous recombination site at the end of the homology shared between the two *neo* segments [Fig. 2B(iv) and 9]. As illustrated in Fig. 9A, the nonhomologous recombination event occurred between two sites, one of which corresponded to a region where the homology between the recombining DNAs terminates. The homology between the two *neo* segments terminates at coordinate 3 (Fig. 9B). One product had recombined coordinate 5 (Fig. 9B), which is only 3 bp away from the end of the homologous region, with coordinate 11627. The two recombining sequences are unrelated except for only 1 bp of homology. In the other product (Fig. 9C), nonhomologous recombination took place exactly at the end of the homology and involved only 3 bp of homology. This type of product is expected to form if the connecting point of some recombination intermediate form takes on an aberrant, recombinogenic structure as it is brought near to the end of the homologous region by branch migration or a similar process (Fig. 1C to G).

Dependence on an *EcoKI* site. To assess the role of *EcoKI* restriction, we removed one of the two *EcoKI* sites [*EcoKI*(2)] from the substrate plasmid (Fig. 2A) (Materials and Methods). This plasmid (pIK151) did not show a high relative transformation efficiency when cleaved with *XhoI* in vitro (Fig. 10A). The level was the same as that found for the negative control (DH10B [*recA1 Δrac r_K⁻ m_K⁻]). Furthermore, the proportion of rearrangement-type plasmids in the transformants was smaller than that with the substrate containing the two *EcoKI* sites (Fig. 10B). We analyzed the structures of some of these rare rearranged-type plasmids with restriction enzymes. All of them lacked the *XhoI* site that had been cleaved before transfer. They appear to have arisen as a result of one nonhomologous recombination event that joined two sites on the parental plasmid. The distribution of the nonhomologous recombination sites was, however, different from that found with the original plasmid as listed in the rightmost two columns of Fig. 7: there was no excess of products involving recombination between the two *neo* segments (type I-III). When pIK43 (two *EcoKI* sites) was prepared in an *EcoKI*⁺ host, the fraction of rearrangement-type plasmids in the transformants (1 of 24) was smaller than with unmodified pIK151, although their transformation efficiencies were nearly the same. The others belonged to the parental type. Therefore, the unmodified state of the *EcoKI*(1) site apparently stimulated this type of illegitimate recombination. Each of these two *EcoKI* sites can stimulate some type of illegitimate recombination when unmodified.*

These results suggest that most of the nonhomologous rearrangement events depend on the presence of an unmodified *EcoKI*(2) site (Fig. 2A). This would be expected if *EcoKI* action initiated at an unmodified *EcoKI* site promoted these rearrangements.

FIG. 6. Dependence of the nonhomologous recombination event on the presence of the homology. (A) pIK43 (*neo* × *neo*) versus pIK32 (*neo*); (B) pIK43 (*neo* × *neo*) versus pIK154 (*neo* × *cml*). A fixed amount of cells from each of the indicated strains was transformed by electroporation with 0.5 μg of pIK43 (*neo* × *neo*), pIK32 (*neo*), or pIK154 (*neo* × *cml*). DNAs were unmodified with *Eco*KI methylase (see Materials and Methods) and were used either intact or after having been cut with *Xho*I. The number of Amp^r transformants from the cut DNA relative to that from the intact DNA was plotted along the horizontal axis. The transformation efficiencies in the strains are as follows (Amp^r transformants from 0.5 μg of cut DNA/Amp^r transformants from 0.5 μg of uncut DNA). (A) BIK1490, 1.00 × 10²/8.6 × 10³ (pIK43), 9.6 × 10²/9.2 × 10³ (pIK32), 2.4 × 10¹/1.14 × 10⁴ (pIK32), and 2.4 × 10¹/1.50 × 10⁴ (pIK32); DH10B, 1.31 × 10⁵/1.02 × 10⁸ (pIK43) and 7.0 × 10⁴/3.0 × 10⁷ (pIK32). (B) BIK1490, 3.0 × 10²/2.4 × 10³ (pIK43), 1.88 × 10¹/1.61 × 10³ (pIK43), 7.5/2.2 × 10³ (pIK154), and 3.8/1.33 × 10³ (pIK154); DH10B, 8.5 × 10⁴/2.9 × 10⁷ (pIK43), 3.9 × 10⁴/2.9 × 10⁷ (pIK154), and 7.0 × 10⁴/2.9 × 10⁷ (pIK154).

DISCUSSION

We have described a novel type of recombination in *E. coli* that depends on an interaction between two homologous DNA segments and that results in illegitimate recombination involving at least one of the two interacting DNAs. The detection of this recombination required that a plasmid unmodified by *Eco*KI be cut with *Xho*I in vitro and transferred to cells with active *Eco*KI. The requirement of at least one of the two *Eco*KI sites on the plasmid was demonstrated.

Mechanism. We have discussed previously various ways by which homologous interaction may lead to nonhomologous recombination (22, 48). One simple explanation is as follows. An intermediate is formed by homologous interaction between two DNAs, and its connecting point migrates along the homologous segment. The connecting point assumes some form of recombinogenic structure, e.g., a strand break, at an end of the homology or within the homology (Fig. 1). The hypothetical structure causes illegitimate recombination between one of the two involved DNA molecules and a third DNA site. One possible form for the connecting point would be a Holliday structure. Although this scheme appears to be in accordance with the presence, in our strain, of a *recG* mutation, which inactivates a branch migrase (37), a *ruvC* mutation, which inactivates a Holliday resolvase (18), and *recBC* mutations, which inactivate a helicase/nuclease (25), further work will be necessary to assess the possible effects of these and other mutations on the frequency and properties of this type of illegitimate recombination.

The nonhomologous recombination that we observed was dependent on the presence of an unmodified *Eco*KI restriction site on the plasmid. *Eco*KI cleaves DNA at a variable distance from the recognition sequence (16). It has been proposed that type I restriction enzymes bind to their recognition sites, trans-

locate DNA toward themselves from both directions, and then cut the DNA where they meet (52). Recent work demonstrated that restriction by *Eco*KI is enhanced by cooperative interactions between target sequences (61). This reaction would explain the need for an *Eco*KI site in our reaction and would suggest that if the translocation of the two enzymes was halted by a Holliday junction, their encounter would result in cleavage of the DNA at this junction. In fact, *Eco*KI can cleave at the base of an immobile Holliday junction in a crude extract (58). This model is also in harmony with the presence of *recG*, *ruvC*, and *recBC* mutations in our strain.

The following synopsis represents one likely course of events (Fig. 11A). Holliday junctions are formed in the region of homology. *Eco*KI molecules bind to both *Eco*KI sites and translocate DNA past themselves until one of them encounters the Holliday junction, where it is stalled and awaits the arrival of the second *Eco*KI molecule. The Holliday junction might be

FIG. 7. Classification of the nonhomologous recombination products. (A) Four regions of pIK43. I, II, III, and IV in panel A define the upper *neo* repeat, the right unique sequence, the lower *neo* repeat, and the left unique sequence, separated by the four restriction enzyme sites. K(1) and K(2) indicate the two *Eco*KI recognition sites. pIK151 lacks part of segment II including the K(2) site (see Materials and Methods). Some *Eco*KI and *Hind*III sites are omitted. (B) Classification. Plasmid DNA was recovered from each of the Amp⁺ colonies of BIK1490 (*recB21 recC22 Δrac recG258 ruvC53 rK*⁺ *mK*⁺) that had been transformed with pIK43 cut with *Xho*I or pIK151 cut with *Xho*I (see legends to Fig. 3 and 9). The DNA was digested with one of four restriction enzymes, *Eco*KI, *Hind*III, *Eco*RV, and *Sal*I, to map the nonhomologous recombination sites.

- A (I - I) 5'- AGAAGTAAGGTTCTTCACAAAGATCCGGGACCAAGCGGCCATCGTGCCTCCCACTCCTGCAGTTCGGGGGCATGGATGCGCGGATAGCCGCTGCTGG -3'
5'- GCCGGAGAACCTGCGTGCAATCCATCTTGTTCATCATGCGAAACGATCCTCATCCTGCTCTTGATCAGATCTTGATCCCCTGCGCCATCAGATCCTTG -3'
5'- AGAAGTAAGGTTCTTCACAAAGATCCGGGACCAAGCGGCCATCGTGCCTCATCCTGCTCTTGATCAGATCTTGATCCCCTGCGCCATCAGATCCTTG -3'
- B (I - II) 5'- GGGGGCATGGATGCGCGGATAGCGCTGCTGGTTTCTGGATGCCGACGCTATTTCGACTGCCGGTAGAACTCCGCGAGGTGCTCCAGCCTCAGGCAGCAG -3'
5'- CTATCTAAGAGCTGAAACACAAGCATTAAAGCATGCCTGCATATATTAAGCTAGGTGCAAGCTGGCAACTGGGGAAGGAAGCTGGAAGTCTATCCTAACT -3'
5'- GGGGGCATGGATGCGCGGATAGCGCTGCTGGTTTCTGGATGCCGACGCTATTTCGACTGCCGGTAGAACTCCGCGAGGTGCTCCAGCCTCAGGCAGCAG -3'
- C (I - II) 5'- TATTACTAAACACAGCATGACTCAAAAACTTAGCAATTCGAAGGAAAGTCTTGGGGTCTTCTACCTTCTCTCTTTTGGAGGAGTAGAATGTTG -3'
5'- TTGTTTATTGGAACACACCCGGTACACATCCTGCTCCAGCATTTGCAGTCCGTGATTGAATTATTGTCTGGCTAGACTTCATGGCGCTGGCACCCGAA -3'
5'- TATTACTAAACACAGCATGACTCAAAAACTTAGCAATTCGAAGGAAAGTCTTGGGGTCTTCTACCTTCTCTCTTTTGGAGGAGTAGAATGTTG -3'
- D (I - II) 5'- AAACAGGTTTTCTCATTAAAGGCATTCCACCCTGCTCCCATTCATCAGTCCATAGGTTGGAATCTAAAATACACAAACAATTAGAATCAGTAGTTTA -3'
5'- GGGAGCCTGGGGACTTTCCACACCTGGTTGCTGACTAATTGAGATGCATCTTTCATACATCTTGCCCTGCTGGGGAGCCTGGGGACTTTCCACACCCTAA -3'
5'- AAACAGGTTTTCTCATTAAAGGCATTCCACCCTGCTCCCATTCATCAGTCCATAGGTTGGAATCTAAAATACACAAACAATTAGAATCAGTAGTTTA -3'
- E (I - III) 5'- AGCAAGCAAGAGTTCTATTACTAAACACAGCATGACTCAAAAACTTAGCAATTCGAAGGAAAGTCTTGGGGTCTTCTACCTTCTCTCTTTTGG -3'
5'- TCAGCTGCTGCTGAGGCTGGACGACCTCGCGGAGTTCTACCGGCAGTGCATATCCGTCGGCATCCAGGAAACCAGCAGCGGTATCCGCGCATCCATGC -3'
5'- AGCAAGCAAGAGTTCTATTACTAAACACAGCATGACTCAAAAACTTAGCAATTCGAAGGAAAGTCTTGGGGTCTTCTACCTTCTCTCTTTTGG -3'
- F (I - III) 5'- GCATGGATGCGCGGATAGCCGCTGCTGGTTTCTGGATGCCGACGATTTGCATGCGCGTAGAACTCCGCGAGGTGCTCCAGCCTCAGGCAGCAG -3'
5'- CCAAAAAAGAAAGAGTAGAAGACCCCAAGGACTTCTTCAGATTGCTTAAGTTTTTG AGTCATGCTGTGTTTAGTAATAGAACTCTTGCTTGTCT -3'
5'- GCATGGATGCGCGGATAGCCGCTGCTGGTTTCTGGATGCCGACGATTTGCATGCGCGTAGAACTCCGCGAGGTGCTCCAGCCTCAGGCAGCAG -3'
- G (I - III) 5'- ACCACAGAAGTAAGGTTCTTCACAAAGATCCGGGACCAAGCGGCCATCGTGCCTCCCACTCCTGCAGTTCGGGGGCATGGATGCGCGGATAGCCGCT -3'
5'- GGGCGGTTTTATGACAGCAAGCGAACCAGGAATTGCCAGCTGCGGCGCCGCTTCTTTTGTG AAGACCGACCTGTCGGTGCCCTGAATGAAGTGCAGG -3'
5'- ACCACAGAAGTAAGGTTCTTCACAAAGATCCGGGACCAAGCGGCCATCGTGCCTCCCACTCCTGCAGTTCGGGGGCATGGATGCGCGGATAGCCGCT -3'
- H (I - III) 5'- CCTTCTCTCTTTTGGAGGAGTAGAATGTTGAGAGTCAGCAGTAGCTCATCATCACTAGATGGCATTTCTCTGAGCAAAACAGGTTTCTCATT -3'
5'- CCATACCACATTTGTAGAGGTTTACTTGTCTTAAAAACCTCCACACCTCCCTGAACTGAAACATAAAATGAATGCAATTGTTGTTAACTTG -3'
5'- CCTTCTCTCTTTTGGAGGAGTAGAATGTTGAGAGTCAGCAGTAGCTCATCATCACTAGATGGCATTTCTCTGAGCAAAACAGGTTTCTCATT -3'
- I (I - IV) 5'- CATAGTTATTAATAGCAGACACTCTATGCCTGTGTGGAGTAAGAAAAAGCAGTATGTTATGA TATACTGTTATGCCTACTTATAAGGTTACAGAATA -3'
5'- ACGATGATCGGCCTGTGCTTGGGATTCGGAATCTTGACGACCTTCTCAAGCCTTCGCTGCTGCTCCGCCACCAACGTTTCGGCGAGAAGCAGG -3'
5'- CATAGTTATTAATAGCAGACACTCTATGCCTGTGTGGAGTAAGAAAAAGCAGTATGTTATGA TATACTGTTATGCCTACTTATAAGGTTACAGAATA -3'
- J (IV - I) 5'- TTATTGAAGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGATTATGA AAATAACAAATAGGGGTTCCGCGCACATTTCCCGA -3'
5'- GGTGAGATGACAGGAGATCTGCCCCGCGACTTCGCCAATAGCAGCAATTCCTCTCCCGCTT CAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCC -3'
5'- TTATTGAAGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGATTATGA AAATAACAAATAGGGGTTCCGCGCACATTTCCCGA -3'

FIG. 8. Sequences of several nonhomologous recombination joints. The DNA sequences at the sites of nonhomologous recombination in plasmid DNA from 10 of the BIK1490 (*recB21 recC22 Δrac recG258 ruvC53 r_K⁺ m_K⁺*) *Amp^r* colonies transformed with pIK43 cut with *Xho*I (experiment 1 in Fig. 7) were determined after subcloning into pUC118 or pUC119 (Materials and Methods). The sequences were compared with that of the parental plasmid pIK43 so as to deduce the recombination sites. The numbers correspond to the coordinates in pIK43 (Fig. 2A).

pushed to the end of homology by the first *Eco*KI molecule. When they collide, they cleave off one or more arms of the Holliday junction and produce a double-strand break at the ends of the homology or in the middle of the homology. This double-strand break then stimulates illegitimate recombination with a third site. It is natural to invoke involvement of a third site because the illegitimate joining sites lie far from each other in all the products. If only homologous interacting sites were involved, the joining sites would be at the same locus in the *neo* repeats. Further analysis using *Eco*KI restriction enzyme mutants and plasmid substrates with defined arrangements of wild-type and/or mutated *Eco*KI sites will be required to further elucidate the role of *Eco*KI restriction in this phenomenon.

This nonhomologous recombination was detected at a high frequency in BIK1490 (*recB21 recC22 Δrac recG258 ruvC53 r_K⁺ m_K⁺*) but not in the corresponding parental strain BIK1489 (*recB21 recC22 rac⁺ sbcA23 recG258 ruvC53 r_K⁺ m_K⁺*). This difference can be explained by excision of the *Rac* prophage. First, the restriction alleviation gene (*lar*) on the *Rac* prophage inhibits K-12 restriction (20) and therefore, presumably, restriction-dependent illegitimate recombination. Second, the homology-dependent double-strand break repair reaction with our substrates needs the *recE* and *recT* genes on the *Rac* prophage (33, 54).

The presence of two copies of a long sequence in inverted orientations is known to stimulate illegitimate recombination

FIG. 9. Nonhomologous recombination at an end of the homology. (A) Scheme. The middle DNA has sequence homology with the upper DNA. Exactly at the boundary of this homology, the middle DNA experiences nonhomologous recombination with the lower DNA. (B) One example. This clone belongs to type I-III in experiment 1 from pIK43 in Fig. 7. The recombination itself involves only 1 bp of homology (underlined). The numbers correspond to the coordinates in the parental plasmid pIK43 (Fig. 2A). See Fig. 2B(iv) for the overall configuration of the product. (C) Another example. This clone belongs to type I-III in experiment 1 from pIK43 in Fig. 7. The recombination involves only 3-bp-long homology (underlined).

FIG. 10. Effect of deletion of an *Eco*KI restriction site. (A) Transformation efficiency. Fixed amounts of cells from each of the indicated strains were transformed by electroporation with 0.5 μ g of pIK43 [*Eco*KI(1)⁺/*Eco*KI(2)⁺] or pIK151 [*Eco*KI(1)⁺/*ΔEco*KI(2)] DNA unmodified with *Eco*KI methylase (see Materials and Methods), either intact or after having been cut with *Xho*I. The number of Amp^r transformants from the cut DNA relative to that from the intact DNA is shown along the horizontal axis. The transformation efficiencies in the bacterial strains are as follows (Amp^r transformants from 0.5 μ g of cut DNA/Amp^r transformants from 0.5 μ g of uncut DNA): BIK1490, $1.63 \times 10^3/1.15 \times 10^3$ (pIK43), $2.5 \times 10^3/1.55 \times 10^3$ (pIK43), $6.1 \times 10^4/4.2 \times 10^4$ (pIK151), and $6.4 \times 10^4/2.8 \times 10^4$ (pIK151); DH10B, $2.1 \times 10^3/1.26 \times 10^3$ (pIK43), $8.5 \times 10^3/2.9 \times 10^3$ (pIK43), $5.4 \times 10^5/2.9 \times 10^5$ (pIK151), and $2.0 \times 10^5/1.20 \times 10^5$ (pIK151). (B) Classification of the products. Plasmid DNA was recovered from the Amp^r colonies after transformation with cut DNA and was classified into the nonhomologous recombination type and the parental type by digestion with *Xho*I and *Eco*RI. n, number of transformant colonies analyzed.

in other systems (13, 35). It was suggested that this stimulation results from strand slippage during replication, resulting in the formation of hairpin-like secondary structures at the inverted repeats—in a sense, homologous interaction. In our system, such replication slippage is unlikely to be the cause of illegitimate recombination because the ColE1 origin requires superhelicity for replication initiation (17, 43) and our substrate

DNA lacked superhelicity because of double-strand breakage. Even in the cited experiments, homologous interaction between the repeats or protrusion of the palindromes may have resulted in a Holliday structure or some other recombinogenic structure.

In all of the products analyzed by restriction enzyme cleavage, the illegitimate recombination that we observed can be

FIG. 11. Possible roles and consequences of homology-associated nonhomologous recombination. (A) Exclusion of non-self DNA, illustrated specifically for *Eco*KI-mediated illegitimate recombination described in this work. Shown is a possible role of the *Eco*KI enzyme in defense against foreign DNA. Foreign DNA tries to replace homologous recipient DNA by homologous recombination. A Holliday junction is formed. *Eco*KI enzyme recognizes unmodified *Eco*KI sites on the invading DNA, starts translocating along the DNA, and becomes stalled at the Holliday junction. The two *Eco*KI enzyme molecules meet at the junction and introduce a double-strand break there. This will abort homologous recombination, kill the donor DNA, and kill the recipient in most cases. The double-strand break stimulates illegitimate recombination. A very small fraction of the products will be viable. (B) Highly homologous members of a repetitive gene family at different locations. Nonhomologous recombination near the boundary of the repetitive sequences (or their diversified sequences) may destroy the genome and kill the cell. This may rarely result in a genome carrying one copy of the repeated sequence truncated near its end at the site of nonhomologous rearrangement. (C) A gene and a highly homologous gene with a heterologous insertion. Homologous pairing generates an intermediate that moves along the homologous region by branch migration until it encounters heterology. This results in nonhomologous rearrangement with a third, unrelated DNA. In most cases, the rearranged genome is inviable, and this leads to killing of the cell. Only in rare cases will the rearranged genome remain intact and not kill the cell.

most easily explained as the joining of one site with another site within the same molecule. We do not know, however, whether more than one molecule is required for each of these events.

Possible roles and consequences. The phenomenon described here occurred in a strain with multiple mutations in recombination-related genes. Further experiments will be necessary to see whether this type of recombination also takes place in *E. coli* strains with different genetic backgrounds.

What might be the roles and consequences of this novel reaction in *E. coli*? This reaction might be a manifestation of the resistance of the *EcoKI* system to the incorporation of unmodified, foreign genes into a chromosome by homologous recombination as illustrated in Fig. 11A. According to this scheme, homologous pairing between unmodified, foreign DNA and recipient DNA in the chromosome would generate an intermediate form such as a Holliday structure. *EcoKI* enzymes bound to the unmodified *EcoKI* sites on the donor DNA would eventually introduce a double-strand break at the connecting point, resulting in aborted homologous replacement by the foreign DNA. Type I restriction indeed alters the recombination patterns of homologous recombination in transduction with replaced segments of DNA becoming shorter and being multiplied in the presence of restriction (40).

If the recipient chromosome (as opposed to the invading chromosome) is cut by the restriction enzyme, this could represent suicidal defense against invasion. Because of such cell suicide, the neighboring clonal cells might evade infection with this invading genetic element. This type of suicidal defense is well known in the case of viral infection in bacteria (phage exclusion) (51) and in mammalian somatic cells. Without such cell suicide, all the members of a clone might be eliminated by the secondary infection with the invader. Indeed, type II restriction-modification gene pairs program suicidal defense against invasion of an incompatible plasmid (41) or of a homologous DNA (21, 41a). When a restriction-modification gene pair is displaced by a homologous DNA or an incompatible plasmid, the descendant cells will gradually die because they will retain less and less of the modification enzymes and the numerous recognition sites on the chromosome will be exposed to attack from the residual restriction enzyme activity. This reaction is also similar to the mismatch recognition system with respect to the role it plays in the exclusion of heterologous DNA (45).

Illegitimate recombination might represent a secondary consequence of restriction breakage. This would have the consequence of generating diversity.

Related phenomena and some generalizations. A similar joining event in yeast cells has been described. It requires DNA ends and sequence homology, and the joining sites display a few nucleotides of homology (26–30). In maize, the daughters of a broken chromosome can be fused at the site of breakage and form an inverted duplication. This initiates the breakage-fusion-bridge cycle (39). Some aberrant products in mammalian gene targeting (3, 9, 22, 48) can be explained by the present mechanisms. There are other lines of evidence supporting the existence of this type of recombination in the genome (22). One is illegitimate recombination between *Alu* repeats, which represent a class of human short interspersed repetitive sequences (22, 42).

What could be the role of illegitimate recombination events dependent on homologous interaction in general? Pairing of repetitive elements such as transposons inserted at different loci might result in illegitimate recombination and destruction of the genome through this mechanism (Fig. 11B). It could pair a chromosome infected with a transposon with an intact sister

chromosome immediately after the insertion (Fig. 11C). Thus, the present nonhomologous recombination mechanism could fight against invasion by transposons and protect the integrity of the genome in the clonal cell population. Very rarely this reaction might generate a viable genome with a nonhomologous rearrangement (Fig. 11). These might be the nonhomologous recombinants observed with transposons (22, 42). Thus, this mechanism could ensure genome stabilization within short time scales and accelerate genome destabilization over long evolutionary periods. This is similar to the role proposed for restriction (2). We do not know what agent plays a role of restriction enzyme in other systems.

Homologous interaction, defined as an interaction between two DNAs based on their sequence homology, appears to be an essential property of DNA. Recent work has revealed the presence of various manifestations of homologous interaction other than classical homologous recombination. These include the silencing of genes by methylation (46), destruction of genes by intensive point mutagenesis (50), gene regulation by the transvection effect (44, 57), and silencing of genes by some unidentified mechanism in plant and animal cells (21, 38). The present homology-associated illegitimate recombination mechanism could be similar to these homology-dependent gene inactivation mechanisms. They might have a role for antirepeats (22), and there is evidence for this hypothesis in the case of repeat-induced point mutations (19). Even some forms of homologous recombination can be regarded as a gene destruction mechanism (22, 56). Homologous interaction might represent one type of competitive exclusion between homologous genes. These ideas will be treated in more detail elsewhere (22, 53).

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