DNA Inversions Between Short Inverted Repeats in *Escherichia coli*

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ABSTRACT

Using site-specific mutagenesis *in vitro*, we have constructed *Escherichia coli* strains that allow the detection of the inversion of an 800-bp segment in the *lac* region. The invertible segment is bounded by inverted repeats of either 12 or 23 bp. Inversions occurring at these inverted repeats will restore the Lac$^+$ phenotype. Inversions can be detected at both short homologies at frequencies ranging from 0.5×10^{-8} to 1×10^{-7}. These events, which have been verified by DNA sequence analysis, are reduced up to 1000-fold in strains deficient for either RecA, RecB or RecC. They are not reduced in strains deficient in the RecF,J pathway. These results show that the RecB,C,D system can mediate rearrangements at short sequence repeats, and probably plays a major role in cellular rearrangements.

GENETIC rearrangements play a central role in many biological processes, including development (BOSTOCK 1984), control of gene expression (HABER 1983; SILVERMAN and SIMON 1983; GOLDEN, MULLIGAN and HASELKORN 1987; STRAGIER *et al.* 1989), antibody formation (TONEGAWA 1983), and evolution (RILEY and ANILIONIS 1978). Studies with oncogenes have strongly implicated genetic rearrangements in the origin of human cancers (LEDER *et al.* 1983; MARX 1984). In bacteria, many rearrangements occur at short, imperfect homologies. For instance, spontaneous deletions in bacteria and bacteriophage occur preferentially at short repeated sequences that are often less than 10 bp (FARABAUGH *et al.* 1978; STUDIER *et al.* 1979; PRIBNOW *et al.* 1981; ALBERTINI *et al.* 1982). Whereas some duplications occur at large homologies and are dependent on *recA* (ANDERSON and ROTH 1981), others occur at short homologies (EDLUND and NORMARK 1981; WHORISKEY *et al.* 1987). Several human diseases have been shown to result from rearrangements at short sequence repeats that are strikingly similar to those detected in bacteria. Some examples are Kearns-Sayre/chronic external opthalmoplegia plus syndrome (JOHNS *et al.* 1989; SCHOFFNER *et al.* 1989), Fabry disease (KORNREICH, BISHOP and DESNICK 1990), angioedema (STOPPA-LYONNET *et al.* 1990), and Duchenne muscular dystrophy (DARRAS *et al.* 1988). Clearly, it is important to understand the pathways that lead to different kinds of genetic rearrangements.

The finding that many rearrangements occur preferentially at short homologies has led to two basic models for their formation (FARABAUGH *et al.* 1978; ALBERTINI *et al.* 1982; see discussion in WHORISKEY, SCHOFIELD and MILLER 1991), that invoke either slipped mispairing during replication, or recombination events as being the prime cause of such rearrangements. Experimental evidence in favor of each mechanism has been presented (*e.g.*, SINGER and WESTLYE 1988; DASGUPTA, WESTON-HAFER and BERG 1987). It is likely that deletions and duplications at short repeated sequences occur via both types of pathways, "replicative" and "recombinational." Recombination pathways might involve some of the known Rec systems (see review by SMITH 1989), or be dependent on DNA gyrase (IKEDA 1986). The nature of the replicating element, the experimental conditions, and the DNA sequences surrounding the short homologies all serve to dictate the predominant mechanism.

In order to determine the role that recombination might play in generating rearrangements at short homologies in *E. coli*, we have constructed a system which detects inversions of a segment of DNA flanked by short inverted repeats. Inversions (see Figure 1) cannot result from replicative slippage mechanisms, and require the equivalent of a reciprocal recombination event, either by a single reciprocal recombination, or by two or more nonreciprocal recombination events (see Discussion in MAHAN and ROTH 1988). Can inversions occur at short inverted homologies, and will they be affected by different *rec* alleles? Here we show that such inversions do occur and are in fact greatly reduced in the absence of a functioning RecB,C,D system. These results argue for a key role of the RecB,C,D system in promoting rearrangements in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and reagents: F′ *lacpro* episomes were in the S90C strain background, *ara (gpt-lac)5 rpsL*. P1 transduction (MILLER 1972) was used to construct *recB*, *recC*, *recJ*

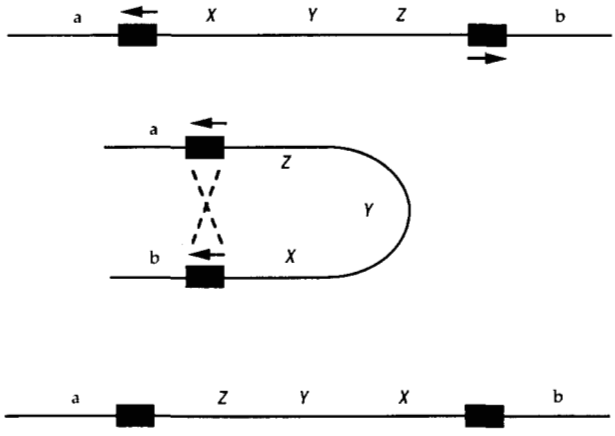

FIGURE 1.—Inversions at inverted repeats. The inversion of the segment between the inverted repeats occurs after a reciprocal recombination event, as shown here.

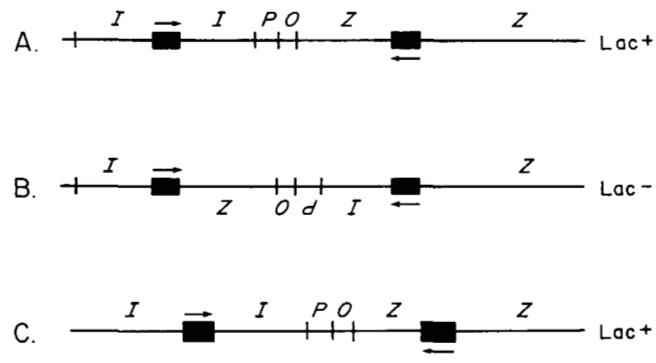

FIGURE 2.—System for inversion. The creation of inverted repeats in *lacZ* and *lacI*, together with silent restriction sites (A), permits the inversion of an 800-bp segment of *lac*, which results in the Lac$^-$ phenotype (B). Revertants to Lac$^+$ can occur by an inversion at the repeated sequences (C).

and *topB* derivatives from strains carrying transposons inserted into the *rec* or *top* gene.

***In vitro* mutagenesis:** An f1 phage containing a 1.7-kb insert (MOTT, VAN ARSDELL and PLATT 1984) was used as template for site-directed mutagenesis using mutant oligonucleotides as described by ZOLLER and SMITH (1982). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer, purified and kinased as described (NORMANLY *et al.* 1986). To identify the phage containing the correct mutation, the mutant oligonucleotides were 5′ end-labeled with [^{32}P]ATP (WHORISKEY *et al.* 1987) and used to probe the mutagenesis mixture by plaque hybridizations (HANAHAN and MESELSON 1980) on nylon filters (Gelman Sciences). All potential candidates were plaque purified twice prior to dideoxy sequencing by the method of SANGER, NICKLEN and COULSON (1977).

Isolation of Lac$^+$ revertants: Five overnight cultures of strains grown in LB medium (MILLER 1972) were plated on lactose-minimal plates (five samples of each culture) and titered on glucose-minimal plates. Colonies were counted and picked for purification after 36 hr.

Southern analysis: Total *E. coli* chromosomal/episomal DNA was prepared from cultures grown overnight. Oligonucleotides were 5′ end-labeled and purified as described (WHORISKEY *et al.* 1987). An aliquot of 10 μg of DNA was digested with the appropriate enzyme, separated by electrophoresis on 1% agarose gels (Bio-Rad) in Tris-borate, EDTA buffer (DAVIS *et al.* 1980) at 100 V for 5 hr. Southern analysis on nylon membranes (Gelman) was as described by SOUTHERN (1975).

Polymerase chain reaction (PCR) amplification: Chromosomal DNA was amplified by the PCR as follows. One microgram of chromosomal DNA was mixed with 30 pmol of each 3′ and 5′ oligonucleotide, 5 units of *Taq* polymerase (U.S. Biochemical Corp.), and then subjected to 40 cycles (95°, 40 sec; 59°, 1 min; 72°, 1 min) of amplification in 50 mM KCl, 50 mM Tris-HCl, 4 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, and 3.0 mM each dATP, dCTP, dGTP and dTTP. The amplified DNA was isolated from an agarose gel and sequenced as described by KRAFT *et al.* (1988).

RESULTS

Experimental design: The goal is to construct a system in which inversions can be monitored in a simple and straightforward manner. If we invert a region of the *lac* operon including a significant portion of *lacZ* (Figure 2), then the Lac$^-$ phenotype will result. Only the correct inversion back to wild-type will regenerate the Lac$^+$ phenotype, as shown in Figure 2. By placing a silent restriction site in *lacZ* (Figure 3), and engineering sequences in *lacI*, we can create an invertible region with varying amounts of an inverted repeat (IR), as shown in Figure 4, which indicates the inverted repeats of 12 and 23 bp that we designed for this study. (It should be noted that there is an additional potential IR of CCATT, which is flush with the left IR, as shown in Figure 3, and 1 bp from the right IR, in each case. This may increase the effective size of the inverted repeat by up to 5 bp). We end up with a plasmid and an episome containing a *lac* operon in which the sequence between bp 648 and 1445 (numbering as in SILHAVY, BERMAN and ENQUIST 1984) is inverted. The construction strategy is outlined in the following steps.

1. A *Hin*dIII site was introduced (Figure 3) at coding positions 52/54 in *lacZ* (bp 1445 of the *lac* region; SILHAVY, BERMAN and ENQUIST 1984) by site-directed mutagenesis of phage f1R1.7 (MOTT, VAN ARSDELL and PLATT 1984), which contains 1.7 kb of the *lac* operon, including all of *lacI*, *P* and *O*, and the first 440 bp of *lacZ*, to generate f1R1.7 *Hin*dIII (Figure 3A).

2. The inverted repeats of *lacZ* sequence were created in *lacI* by site directed mutagenesis. The inverse complement of 12 or 23 bp of *lacZ*, composed of the *Hin*dIII site and either 6 or 17 bp of 3′ sequence was introduced at position 648 in the *lacI* gene of f1R1.7*Hin*dIII, to generate f1R1.7-112 and -123 (Figure 3, B and C). The 12- and 23-bp inverted repeats replaced *lacI* sequences and resulted in a net gain in the repressor protein of three and one amino acids, respectively. Although the I gene is inactivated, *lacI* gene function is not required for this study.

3. To facilitate future construction of a plasmid containing the entire *lacI* and *lacZ* genes, *Bam*HI sites

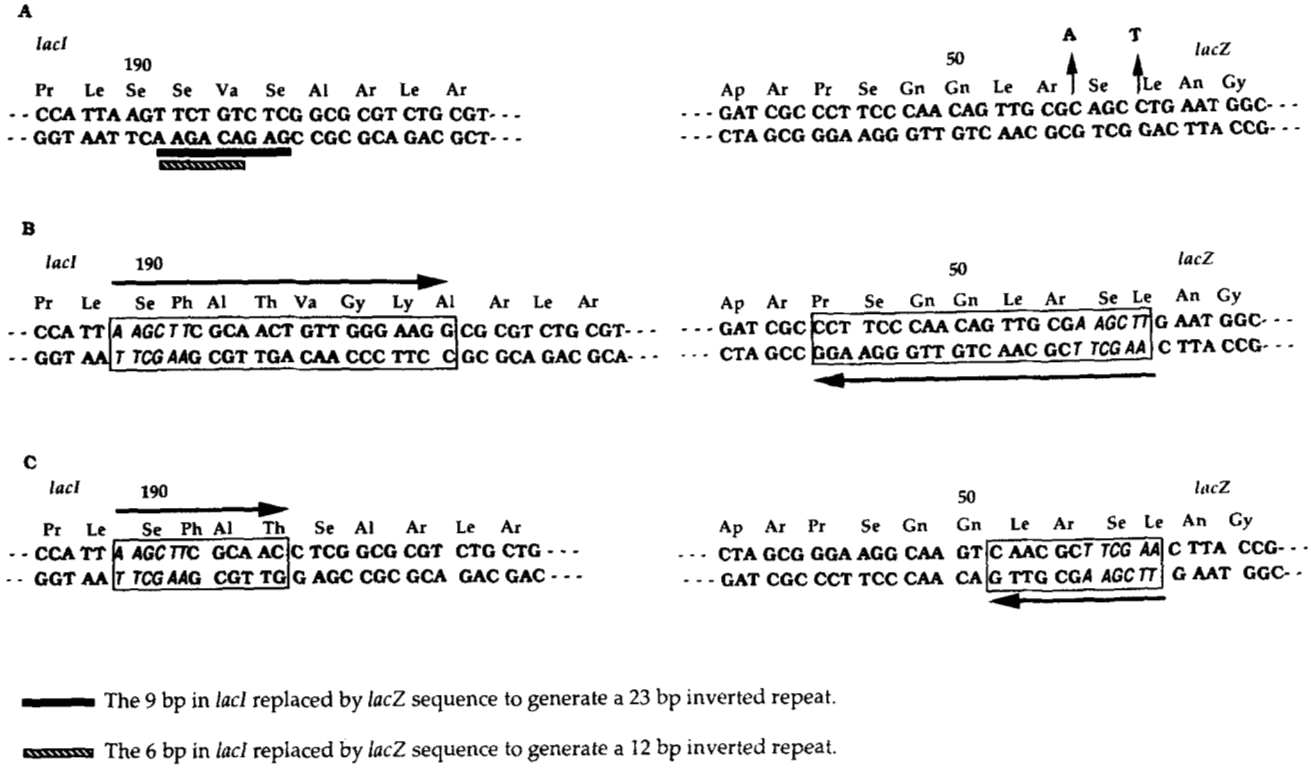

FIGURE 3.—Silent *Hin*dIII site and inverted repeats in *lacI* and *lacZ*. The *Hin*dIII site in *lacZ* (A) and the inverted repeats in *lacI* (B and C) were generated by site-directed mutagenesis. The base changes creating the *Hin*dIII site do not alter the β-galactosidase amino acid sequence. The inverted repeats of 23 bp (B) and 12 bp (C) are boxed; the *Hin*dIII sites are italicized.

were created at codon 95 in the *lacZ* sequence of f1R1.7-112 and -123, and at the analogous position in f1*lacZ*, an f1 phage containing the last 210 bp of *lacI* and the entire *lacZ* gene (Figure 4A; CUPPLES and MILLER 1988). The introduction of the *Bam*HI site results in an insertion of an additional serine in β-galactosidase at coding position 95, which we have subsequently shown to have no effect on the Lac$^+$ phenotype. The *Eco*RI-*Bam*HI fragment of f1R1.7-112 and -123 phage, containing the *lacI* and *lacZ* sequences, was subcloned into pBR329 to generate pBR112 and pBR123. The *Eco*RI fragment of f1*lacZBam* containing *lacI* and *lacZ* was subcloned into the *Eco*RI site of pBR329 to generate pBR*lacZBam* (Figure 4B).

4. To invert the DNA between the *Hin*dIII sites, pBR112 and pBR123 were digested with *Hin*dIII, religated and screened by restriction analysis for those that contained the 800-bp *Hin*dIII fragment in opposite orientation. Plasmids with the inverted fragment were designated pBR112-I and pBR123-I (Figure 5).

5. To generate a plasmid containing the entire *lacI* and *lacZ* genes, the *Eco*RI-*Bam* fragments of pBR112-I and pBR123-I were ligated to the *Bam*HI-*Eco*RI fragment of f1*lacBam* (described above) and subcloned into the *Eco*RI site of pBR329. Subclones containing the entire *lacI* and *lacZ* genes were designated pBR212 and pBR223 (Figure 5). The proper orientation of the fragments was verified by restriction analysis.

6. To derive an episome with the inverted *lacI-lacaZ* segment, the plasmids pBR212 and pBR223 were transformed into a Lac$^+$ strain containing an F′ *lacpro* episome. The episome was transferred by conjugation to a strain (S90C) deleted for the *lacpro* region, selecting for Pro$^+$, and rare (less than 1%) Lac$^-$ derivatives were detected on indicator plates with Xgal (MILLER 1972). The presence of the inverted *lacI-lacZ* segment on the episome was verified by Southern analysis. The integrity of the junction sequences containing the 12- or 23-bp inverted repeats was verified by PCR amplification of the DNA between bp 290 and 1535 and directly sequencing the amplified DNA. Figure 6 (top) depicts the sequenced junctures of the recombined episome.

Inversions at short inverted repeats: We have used the strains constructed in the previous section to monitor the level of inversions that occur at inverted repeats of 12 and 23 bp. Only inversions at these repeats can restore the Lac$^+$ phenotype (see Figure 2). Table 1 shows the Lac$^+$ revertant frequencies for strains CC215 and CC216, which carry a F′ *lacpro* episome with the *lacI-Z* inversion flanked by 12 and

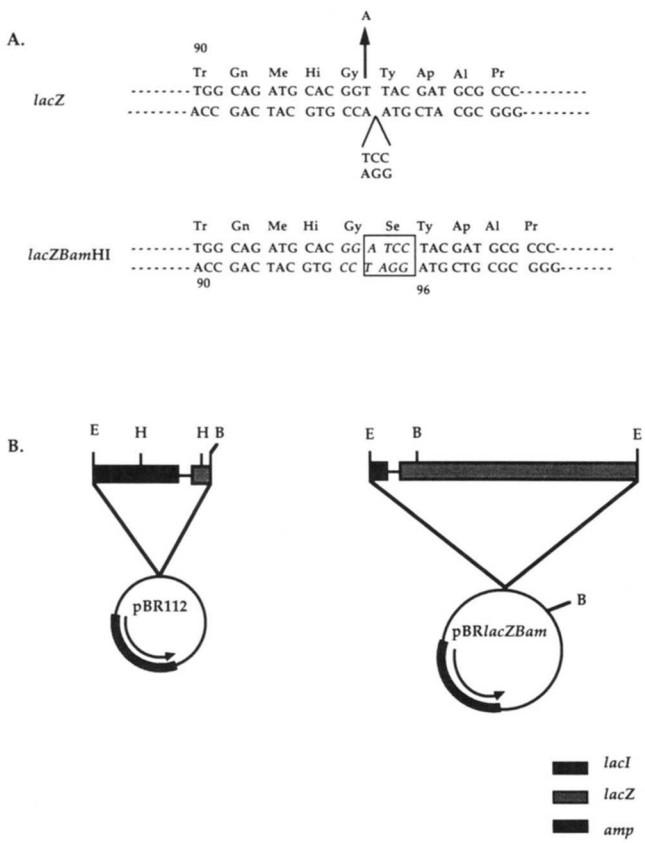

FIGURE 4.—*Bam*HI site in *lacZ*. (A) A *Bam*HI site was generated in *lacZ* at codon 95 in f1R112, f1R123 and f1*lacZBam* by site-directed mutagenesis. The mutagenesis resulted in an insertion of an additional serine at amino acid position 95. (B) The *Eco*RI-*Bam*HI *lacI-Z* fragment of f1R112 and f1R123 was cloned into pBR329 and the *Eco*RI *lacI-Z* fragment of f1*lacZBam* was cloned into the *Eco*RI site of pBR329 to generate pBR112, pBR123 and pBR *lacZbam*, respectively. **B**, *Bam*HI; **E**, *Eco*RI; **H**, *Hin*dIII.

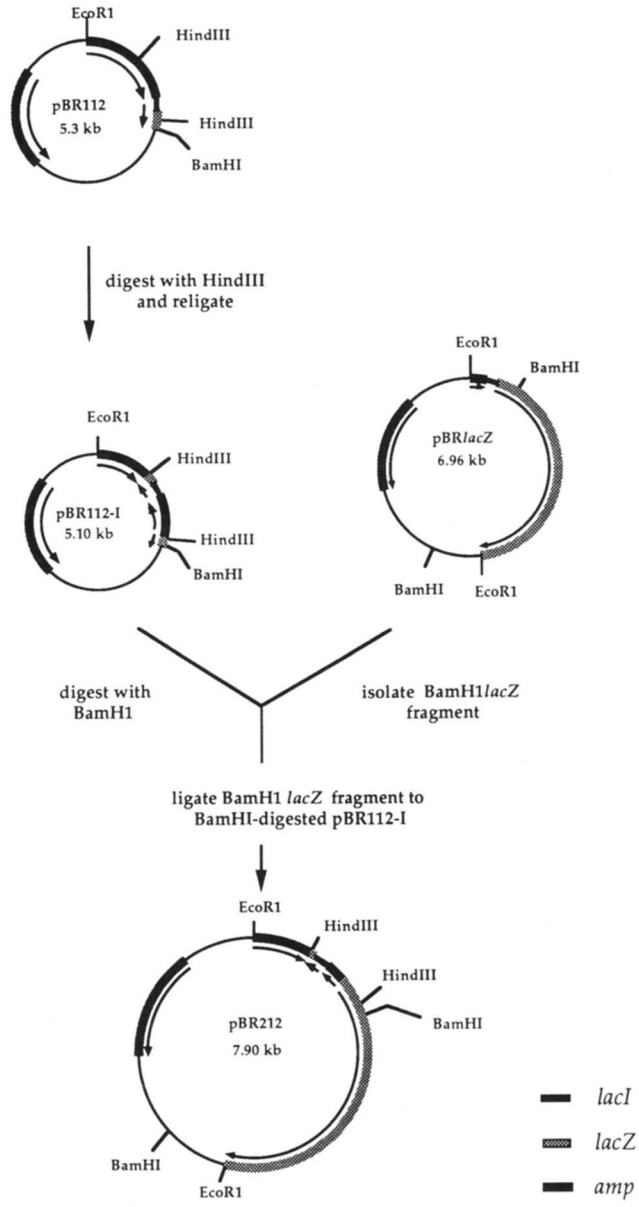

FIGURE 5.—Construction of plasmid containing all of *lacI* and *lacZ*. (A) the *Hin*dIII *lacI-Z* fragment was inverted by digesting pBR112 and pBR1123 with *Hin*dIII and religating the vectors and their respective fragments. The orientation of the *Hin*dIII fragment was verified by restriction analysis. Those closes with the fragment in the opposite orientation were designated pBR112-I and pBR123-I. (B) Thje *Bam*HI *lacZ* fragment of pBR*lacZBam* was isolated and ligated to *Bam*HI-digested pBR112-I and pBR123-I to generate pBR212 and pBR223, containing all of *lacI* and *lacZ*.

23 bp, respectively. Revertants carrying inversions at the 12-bp inverted repeat occur in the population at 0.4×10^{-8}, and at a 24-fold higher rate when the inverted repeats are 23 bp, as shown in Table 1.

The orientation of the *Hin*dIII fragment in 8 Lac$^+$ revertants of CC215 (12-bp inverted repeats) and 8 Lac$^+$ revertants of CC216 (23-bp inverted repeats) was determined by Southern analysis. A Southern blot of DNA from the starting Lac$^-$ strains and Lac$^+$ revertants, digested with *Eco*RV, which cuts asymmetrically within the *Hin*dIII fragment, was hybridized to an oligonucleotide that hybridizes to the inverted DNA (probe 23, while a 1.5-kb fragment is detected if the fragment is reinverted (Lac$^+$). In all cases, a 1.5-kb fragment was detected in the DNA from the Lac$^+$ revertants. The digested DNA was also hybridized to an oligolabeled probe of all of *lacI* and *lacZ*. The chromosomal DNA fragments detected by the oligolabeled probe indicated that the *lacI*-lacZ DNA did not undergo any gross rearrangements other than the inversion of the fragment between the repeats.

The integrity of the junctions of the inverted *Hin*dIII fragment was verified by PCR amplifying the DNA between nucleotides 378 and 1567 and then sequencing across the junctions. The sequences across the 3′ and 5′ junctions of eight Lac$^+$ revertants with only the reinverted fragment indicate that the fragment had inverted precisely (see Figure 5, bottom).

A proportion of the Lac$^+$ revertants was unstable, yielding a mixture of Lac$^+$ and Lac$^-$ colonies after passage on nonselective medium. For the 12 bp inverted repeat, approximately 70% of the colonies were initially unstable, whereas for the 23-bp repeat

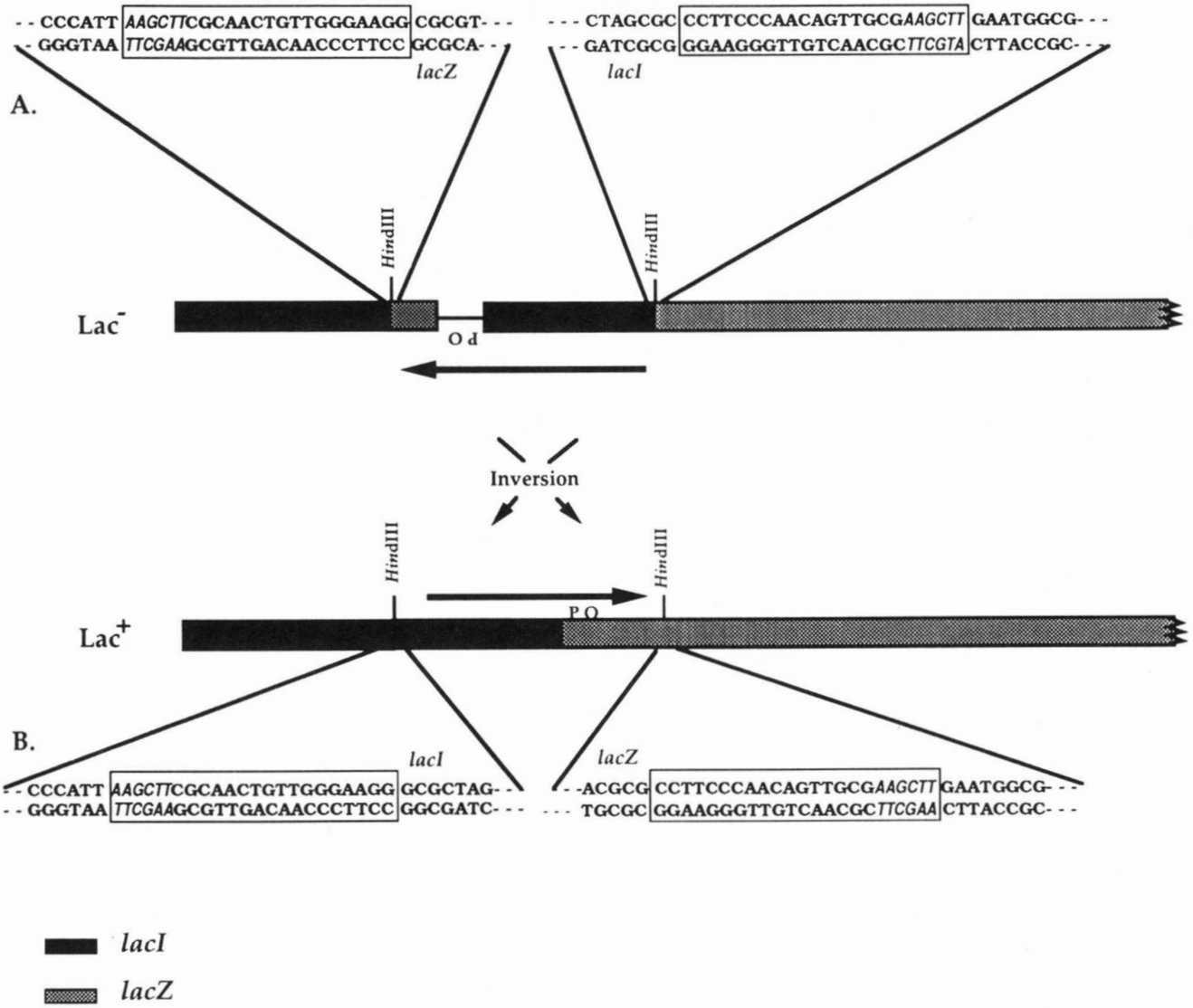

FIGURE 6.—Sequence of the junctions containing the inverted repeats of Lac$^+$ starting strain and Lac$^-$ revertants. (A) The sequence of the *lacI-Z* junctions in the starting Lac$^+$ strain. (B) The sequence of the juctions produced by inversion of the *lacI-Z* fragment flanked by the inverted repeats.

most of the colonies (75%) were stable. Southern analysis of the unstable Lac$^+$ revertants, using DNA from Lac$^+$ strains grown in lactose selective medium, demonstrated the existence of both the 3.0- and 1.5-kb *Eco*RV fragments (see above), suggesting that each of the strains carried both the starting inverted *lac*$^-$ region and the revertant reinverted *lac*$^+$ region. These data are consistent with the unstable revertants carrying examples of two types of F′*lac* episomes which had not yet segregated out, one with the starting *lac*$^-$ region, and the other with the reinverted *lac*$^+$ region.

Inversions in repair-deficient strains: Table 1 also displays the effect of different repair deficient strain backgrounds on Lac$^+$ revertant frequencies. Strains that are either *recA*, *recB* or *recC* display a sharply reduced frequency of inversion formation, with decreases of at least 1,000-fold in some cases. However, strains deficient in the RecF,J recombination system do not appear to be reduced in inversion formation, as evidenced by the data for the *recJ* strain. In fact, there appears to be a slight (2-fold) increase in inversion formation in the *recJ* background. To demonstrate that the reduced frequencies in the *recB* and *recC* strains are not a result of a reduced growth rate, point mutation frequencies have been monitored in the same strains. The frequency of simple frameshifts, as measured by the reversion to Lac$^+$ of *recB* and *recC* strains carrying episomes derived from CC107 and CC109 (CUPPLES *et al.* 1990), is not greatly affected, as is shown in Table 2.

DISCUSSION

We designed a system to test for inversions at short, inverted repeats. Lac$^-$ cells can be restored to Lac$^+$ by inverting an 800-bp segment at inverted repeats of

TABLE 1

Lac$^+$ revertant frequency resulting from inversions

Strain background	Revertant frequency ($\times 10^8$) with length of inverted repeats at 23 bp	12 bp
Wild type	9.6 ± 1.2	0.4 ± 0.2
recA	≤0.01	≤0.01
recB	0.3 ± 0.2	—
recC	≤0.01	—
recJ	19.0 ± 0.5	—

Samples from five or more overnight cultures of each strain were plated on minimal lactose plates, and the number of colonies scored after 36 hr. The average revertant frequency was determined by the number of colony-forming units per ml on lactose plates divided by the number of colony forming units per ml on glucose plates. Entries given as — indicate not tested.

TABLE 2

Lac$^+$ revertants resulting from frameshift mutations

Strain background	Revertant frequency ($\times 10^8$) with strains from which F′*lacpro* is derived CC107	CC109
Wild type	107 ± 15	107 ± 4.8
recB	74.3 ± 6.6	93.0 ± 4.1
recC	64.1 ± 5.2	95.2 ± 1.9

The mutations in CC107 and CC109 (CUPPLES *et al.* 1990) are on an F′*lacpro* episome which was transferred to S90C, and *recB* or *recC* derivatives of S90C. Only specific frameshifts at these sites can restore the Lac$^+$ phenotype. For further details, see legend to Table 1. The mutation in CC107 reverts via the addition of a G:C base bair at a run of 6 Gs, and the mutation in CC109 revertts via the loss of 2 bases (–C–G–) from a run of 5–C–G– repeats (CUPPLES *et al.* 1990).

either 12 or 23 bp. (An additional imperfect inverted repeat of CCATT may increase the effective length of each IR by up to 5 bp.) PCR amplification of selected revertants demonstrates that only the correct inversion can restore the Lac$^+$ phenotype. Lac$^+$ revertants resulting from inversions occur at 0.5×10^{-8} for the 12-bp inverted repeat, and at 1×10^{-7} for the 23-bp inverted repeat. Moreover, the inversions are completely dependent on the RecB,C,D system, since they do not occur or are sharply reduced in strains that are *recA*, *recB* or *recC*. The RecF,J system does not seem to be involved, since inversions are not reduced in a *recJ* strain.

The pathways that lead to rearrangements at short homologies, either direct or inverted, have been under investigation ever since it was recognized that deletions predominate at short homologies (FARABAUGH *et al.* 1978). Evidence has been presented to argue for replicative slippage, recombination, and gyrase-mediated recombination (ALBERTINI *et al.* 1982; DASGUPTA, WESTON-HAFER and BERG 1987; SIGNER and WESTLYE 1988; IKEDA 1986). The work reported here demonstrates that the RecB,C,D system can generate rearrangements at short homologies, in this case inversions at inverted homologies. In fact, the detection of inversions is in of itself an indication of recombination, since an inversion requires either a reciprocal recombination event or two nonreciprocal events (see Discussion in MAHAN and ROTH 1988). Inversions cannot be generated by replicative slippage. These results mirror the findings of MAHAN and ROTH (1989) for inversions at large homologies (approximately 10,000 bp). These inversions are also sharply reduced in *recA*, *recB* or *recC* strains. The short homologies provided by the inverted repeats used in the study reported here are less than the presumed minimal length for RecA promoted recombination [see for instance, GONDA and RADDING (1983) and WATT *et al.* (1985)]. However, these results complement those of SHEN and HUANG (1986), who studied *in vivo* recombination between homologous DNA sequences cloned in phage lambda and a pBR322-derived plasmid by assaying for the formation of phage-plasmid cointegrates by a reciprocal exchange. These authors found that recombination can occur at homologies as low as 19–21 bp, and that the recombination is *recB,C* dependent.

It is of great interest to define additional components of the inversion pathway. What is the effect of each of the known recombination and repair genes on inversion formation? Can certain mutagens stimulate inversions? Do inversions occur at different rates on the chromosome than on the F′ factor, as has been found for certain transposon-excision events (EGNER and BERG 1981; SYVANEN *et al.* 1986)? Also, are there undiscovered genes that are specifically involved in generating inversions? The Lac$^-$ to Lac$^+$ inversion system allows us to look for mutants that increase or decrease inversions by monitoring papillation of indicator medium (NGHIEM *et al.* 1988), in a manner similar to that employed for detecting mutants affected in deletion formation (WHORISHEY, SCHOFIELD and MILLER 1991).

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Note added in proof: HSIEN, CAMERINI-OTERO and CAMERINI-OTERO (1992) report that RecA protein can pair as few as eight bases of homology to form synaptic complexes *in vitro*, strengthening the conclusion that recombination events at short homologies by such proteins can generate rearrangements *in vivo*.

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