The effect of flanking host sequences on the cleavage step of the in vitro Mu DNA strand transfer reaction was investigated. Insertion of a mini-Mu molecule into certain sites in pUC19 results in insertions that demonstrate a decreased ability to form Type 1 complexes in subsequent rounds of transposition. Similarity, changes in the flanking host sequences directly adjacent to the Mu ends by in vitro mutagenesis can also result in Type 1-deficient mini-Mu molecules. Further examination of the inhibition revealed that Type 1 deficient mini-Mu molecules are capable of forming uncut synaptic complexes at normal levels but are compromised in their ability to serve as substrates for phosphodiester bond hydrolysis at the Mu ends. This cleavage defect can be overcome by addition of the Mu B protein and ATP to the reaction. Our data suggest that one of the roles of the B protein may be to provide a mechanism whereby Mu prophages with inhibitory flanking sequences can overcome this obstacle and avoid being trapped at unproductive locations.

The transposition properties of the temperate bacteriophage Mu have been exploited by geneticists, molecular biologists, and biochemists. The ability of Mu to insert its DNA at a high frequency virtually anywhere in the chromosome of Escherichia coli or other hosts has resulted in the use of Mu as a powerful tool for the generation of a variety of genetic rearrangements (Van Gijsegem et al., 1987). Similarly, the very high transposition frequency of Mu has made it invaluable as a paradigm for the study of the mechanisms of DNA rearrangements (Van Gijsegem et al., 1987). Four proteins appear to be involved. The Mu A protein comprises the strand transferase (Maxwell et al., 1987) and binds to defined sequences at each of the Mu ends (Craigie et al., 1984). The E. coli histone-like protein HU (see Drlica and Rouvière-Yaniv (1987) and Schmid (1990)) is required for the reaction (Craigie et al., 1985) and probably functions by increasing the flexibility of the DNA, thereby allowing multiple protein-protein and protein-DNA interactions that would otherwise be energetically prohibited. Integration host factor (IHF) (see Friedman (1986)) is required when the superhelical density of the donor molecule is about -0.025 but is expendable at high levels of supercoiling (Surette and Chaconas, 1989). At high levels of supercoiling, IHF is no longer required, although it stimulates the reaction by decreasing the required concentration of HU by a factor of 4 (Surette and Chaconas, 1989) and by decreasing the required A concentration by a factor of 2. Finally, the Mu B protein, an important component of the multiprotein transposase, has been shown to be involved in a variety of functions, including target DNA capture (Maxwell et al., 1987; Surette et al., 1987; Craigie and Mizuuchi, 1987), transposition immunity (Adzuma and Mizuuchi, 1988), and direct interaction with the Mu A protein to stimulate both strand cleavage (Surette et al., 1991) and strand transfer (Baker et al., 1991; Surette and Chaconas, 1991).

In addition to the protein requirements described, there are three regions of Mu DNA required for the strand transfer reaction: the left end, the right end, and the transpositional enhancer. The left and right ends each contain three Mu A-binding sites (Craigie et al., 1984), and the enhancer (Mizuuchi and Mizuuchi, 1989; Leung et al., 1989; Surette et al., 1989), which is located about 950 base pairs from the left end, also carries several A-binding sites (Craigie et al., 1984) and the IHF-binding site (Surette et al., 1989). Through a complex circuit of protein-protein and protein-DNA interactions, the strand transfer reaction is effected (see Fig. 1) and several stable higher order nucleoprotein complexes are established (Surette et al., 1987; Craigie and Mizuuchi, 1987). These complexes or transpososomes have been the subject of recent footprinting studies that have revealed protection not only of several Mu A-binding sites at the ends (Kuo et al., 1991; Lavoie et al., 1991) but also protection of 10–13 base pairs of flanking DNA beyond the Mu-host junction (Mizuuchi et al., 1991; Lavoie et al., 1991). An altered DNA structure at the Mu-host junction in the Type 1 complex has also been suggested by a dramatic enhancement in hydroxyl radical reactivity on the uncut strand (Lavoie et al., 1991).

It has been generally assumed that sequences outside the ends of a transposon have no effect on the ability of an element to transpose. Because of the nomadic lifestyle of transposable DNA, their perpetuation would be self-limiting if they could be trapped at certain locations. We now report that the flanking host sequences of some mini-Mu insertions can have a dramatic inhibitory effect on the in vitro Mu DNA transposition reaction.

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**Flanking Host Sequences Can Exert an Inhibitory Effect on the Cleavage Step of the in Vitro Mu DNA Strand Transfer Reaction**

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The abbreviations used are: IHF, integration host factor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; DSP, dithiobis(succinimidyl propionate); kb, kilobase pairs.

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2 G. M. Surette and G. Chaconas, unpublished results.
Fig. 1. The in vitro mini-Mu DNA strand transfer reaction. A Type 1 complex or CDC (cleaved donor complex), which is an intermediate in the strand transfer reaction, is formed when a supercoiled mini-Mu donor plasmid is incubated with the Mu A protein and E. coli HU protein. This reaction is stimulated by E. coli IHF protein. The Mu ends are held together in a higher order protein-DNA complex (Type 1 transpososome) defining two topological domains, a relaxed non-Mu vector domain and a supercoiled Mu domain. Disruption of the complex with SDS results in the liberation of a nicked donor plasmid. The Type 2 complex or STC (strand transfer complex) is the product of the strand transfer reaction that remains complexed with protein (Type 2 transpososome). In addition to Type 1 reaction requirements, Mu B protein, ATP, and target DNA are required for Type 2 complex formation. The Type 2 complex can be generated in a single reaction mixture or by conversion of a performed Type 1 into a Type 2 complex. Disruption of the Type 2 complex with SDS liberates the protein-free strand transfer product or θ structure (Craigie and Mizuuchi, 1985; Miller and Chaconas, 1986). This figure is from Surette et al. (1989).

strand cleavage reaction and that the mini-Mu can be rescued from this inhibitory effect by the Mu B protein in the presence of ATP.

**EXPERIMENTAL PROCEDURES**

**DNA Substrates, Reagents, and Enzymes**—The parent mini-Mu donor plasmid pGG215 (7.2 kb) was described previously (Surette et al., 1987). Plasmid pBL10 (6.8 kb) with a mini-Mu in pUC19 was constructed by B. Lavoie in our laboratory. All proteins were purified to ≥95% homogeneity as determined by the presence of a single band by Coomassie Blue staining of SDS-polyacrylamide gels. Purification of the proteins was as described previously in the indicated papers: Mu A protein (Surette et al., 1987; Lavoie et al., 1991), Mu B protein (Chaconas et al., 1988), HU protein (Surette and Chaconas, 1989), and IHF protein (Nash et al., 1987). Protein concentrations were determined as follows: Mu A, absorbance at 280 nm (ε280 = 183) (Kuo et al., 1991); Mu B, absorbance at 280 nm (ε280 = 181) (Chaconas et al., 1988); HU, trichloroacetic acid Lowry assays (Petersen, 1977) using bovine serum albumin standards (values obtained for HU were multiplied by 1.25 to give values consistent with extinction at 280 nm (see Surette and Chaconas, 1989)); IHF, trichloroacetic acid Lowry assays as for HU protein but without any corrections. Taq DNA polymerase was obtained from Pharmacia LKB Biotechnology Inc. Dithiothreitol (sucinimidyl propionate) (DSP) was obtained from Pierce Chemical Co.

**Construction of Plasmids**—Plasmid pWZ10 (10.5 kb) was constructed by cloning an NcoI-AclI mini-Mu fragment (6.5 kb) from pWZ11 into the Smal site of PG82 (4.0 kb) (Baker et al., 1991). pWZ11 (9.8 kb) was generated by inserting a 1.8-kb PatI-PstI fragment containing the chloramphenicol resistance gene excised from the Tn9 transposon into the Mu PstI site of the parent mini-Mu donor plasmid pGG215 (7.2 kb). Insertion and deletion mutants were generated by standard procedures.

**In Vivo Reactions**—In vitro reaction mixtures were carried out as described previously (Surette et al., 1989, 1991). Supercoiled mini-Mu plasmid DNA (75 µg/ml) was incubated with Mu A protein (35 µg/ml), HU protein (15.75 µg/ml), and IHF protein (1.0 µg/ml) in 25 mM Hepes-NaOH pH 7.6, 140 mM NaCl, and 10 mM MgCl2. For Type 1 formation, the above reaction was carried out for 5 min at 30 °C. For Type 2 formation, Mu B protein (30 µg/ml) and ATP (2 mM) were also added to the above reaction, and incubation was performed at 30 °C for 30 min. The reactions were analyzed by electrophoresis in agarose gels, and the extent of reaction was quantified by whole band analysis of fluorescence using a CCD camera and Gel Print Tool Box software from Biophotons Corp., Ann Arbor, MI.

**DNA Sequencing**—Double-stranded DNA sequencing reactions were carried out using the Sequenase Kit from U. S. Biochemical Corp. according to the manufacturer's instructions. The left end sequencing primer OHL1 (20 mer) hybridized to a region in Mu that was 30 bp base pairs away from the Mu left restriction end and to the bottom strand sequence 5′-TTGAGCGTGTATTGAAATG-3′. The right end sequencing primer OHR1 (21 mer) hybridized to a region in Mu that was 100 bp base pairs away from the Mu right end and corresponded to the top strand 5′-GTGATCCCATGTTATAA-3′. Samples were analyzed on 6% sequencing gels run at 50 °C followed by autoradiography.

**Construction of Simple Insertion Library in pUC19**—A Type 1 reaction was performed as described above using pWZ110 as the donor and pUC19 as the target. The reaction was performed and run through a 1 ml Sepharose 6B spin column equilibrated in 10% glycogen. Insertions were recovered and purified as described above. The reaction was then used to transform a recA strain by transformation of gel-eluted simple insertions to eliminate multimeric plasmid forms and cotransforming target DNA.

**Enrichment of Type 1-deficient Simple Insertion DNA**—Type 1 reactions were performed using supercoiled single insertion DNA (6.7 kb) (see details in the legend of Fig. 2) as the mini-Mu donor. The reactions were run in 1% low melting point agarose gels and stained with ethidium bromide. Unrecovered supercoiled DNA was recovered from the gel by phenol extraction followed by ethanol precipitation. The recovered DNA was used to transform a recA strain, and the enriched supercoiled simple insertion DNA was purified by CsCl density gradient centrifugation. The above procedures were repeated several times until a library of DNA that was 70% unrecoverable was obtained.

**Transfer of Type 1-deficient Mini-Mu Molecules into pMK16**—Type 2 reactions were carried out using five different Type 1-deficient simple insertions as the mini-Mu donors and pMK16 (4.5 kb) with a non-Mu donor plasmid KAN attached to it as a target. The reactions were carried out in a low melting point agarose gel and stained with ethidium bromide. Unrecovered supercoiled DNA was recovered from the gel by phenol extraction followed by ethanol precipitation. The recovered DNA was sequenced on a Sequenase kit from U. S. Biochemical Corp. and the library was subsequently transfected to a recA strain by transformation of gel-eluted simple insertions to eliminate multimeric plasmid forms and cotransforming target DNA.

**Phasing for Synapsis and Cleavage**—The conditions used to generate Type 0 complexes were similar to that described above for Type 1 complex, except that 10 mM CaCl2 was used instead of 10 mM MgCl2 (Baker et al., 1991) and incubations were at 30 °C for 60 min. Reactions (25 µl) were then divided in half. To generate a structure, the first half of the mixture (12.5 µl) was treated with 0.5 µl of cross-linker DSP (5 mg/ml, in dimethyl sulfoxide) at room temperature for 15 min; then, 0.5 µl of 0.2 M lysis buffer (pH 8.2) and 1.5 µl of 1 M Tris- HCl (pH 8.2) were added to inactivate the DSP, and incubation was continued at room temperature for 10 min. The restriction enzyme digestions were performed as follows: 3 µl were removed from the cross-linked mixtures and 4.5 µl of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 1 µl of 100 mM MgCl2, and 1.5 µl of 100 mM NaCl) were added to the solution to a final volume of 10 µl. 10 units of enzyme was added per reaction, and incubation was at 30 °C for 60 min. Duplicate reactions were performed and subsequently analyzed in the absence or presence of SDS. HindIII was used to digest the simple insertion DNA library and the Type 1 deficient clones obtained from the library. PstI was used to digest the insertion or deletion constructs and positive controls pGG215 and pBL101.

**Flanking Host Sequences Affect in Vitro Strand Cleavage**

9553
RESULTS

Generation of Type 1-deficient Mini-Mu Insertions in pUC19—As diagrammed in Fig. 2, a library of mini-Mu insertions in pUC19 was constructed using the in vitro Mu DNA strand transfer reaction. The reaction products were deproteinized and used to transform a himA, rec+ recipient strain to select for Mu insertions into pUC19 resulting from cointegrate resolution. A library of 10^6 mini-Mu insertions was generated. The library was used to recover Type 1-deficient mini-Mu insertions by successive rounds of enrichment as follows: mini-Mu plasmid DNA was incubated with A, HU, and IHF to form Type 1 complexes (see Fig. 1). The reactions were run on agarose gels, and the unreacted supercoiled DNA band was excised and used for transformation. This process was repeated until 70% of the DNA was no longer reactive in a standard reaction. The enriched libraries were subsequently plated and individual clones were recovered and analyzed.

Fig. 3 shows restriction endonuclease digestion patterns of the initial and enriched library. Cleavage with MluI, which cuts twice between the Mu ends, resulted in identical digests of the two libraries, indicating that no large scale DNA rearrangements occurred during the enrichment procedure.

The digestion pattern with HindIII, however, which cuts once inside Mu and once in the vector pUC19, the samples were run on a 1% agarose gel and stained with ethidium bromide. S, L, and R denote supercoil, linear, and relaxed forms of pUC19 carrying a simple mini-Mu insertion. The size of the restriction fragment markers is indicated in kilobase pairs. The MluI cleavage pattern does not vary with different sites of insertion, whereas the HindIII cleavage pattern is indicative of the sites at which integration has occurred.

Characterization of Type 1 Reactivity—The ability of the five mapped mini-Mu’s to serve as substrates in Type 1 reactions is shown in Fig. 5A. Relative to the unenriched
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2686 bp

Flanking Host Sequences Close to the Mu Ends Are Responsible for Reduced Type 1 Reaction Efficiency—The five mini-Mu insertions described in Figs. 4 and 5 had an obvious reduction in Type 1 reaction efficiency that resulted from the DNA sequences outside the Mu-host DNA junction. The next question to be addressed was where these inhibitory host sequences were located relative to the Mu ends. Since recent data from two laboratories (Mizuuchi et al., 1991; Lavoie et al., 1991) have established that the Mu A protein in the Type 1 complex protects 10-13 base pairs of flanking DNA beyond the Mu-host junction, we directed our attention to this region. Mini-Mu plasmids carrying mutations in the flanking DNA near the junction were generated. Individual clones were assayed for their ability to form Type 1 complex, and seven Type 1-deficient mini-Mu plasmids were further characterized and sequenced. Fig. 6 shows the reactivity of these mini-Mu's, and Fig. 7 indicates the changes in the flanking sequences. The introduction of small deletions or insertions from two to seven base pairs, respectively, in the host sequences, the five mini-Mu's all showed decreased levels of reactivity. Furthermore, the ability to form a stable Type 1 complex was much more dramatically impaired than the nicking reaction for all five insertions. The spontaneous breakdown of Type 1 complex resulting in the liberation of free nicked DNA has been previously observed when cleavage at one Mu end was inhibited (Surette et al., 1991). Cleavage is normally a concerted event at the two ends, and nicking at both ends is required to stabilize the complex.

To investigate whether the decreased reactivity observed for the Type 1-deficient mini-Mu insertions was a result of the site at which insertion had occurred in pUC19 or whether a defect in the mini-Mu itself was the cause, each of the mini-Mu's was transferred to the vector pMK16. This was done using pMK16 as a target in Type 2 reactions followed by transformation, as described under “Experimental Procedures.” Individual mini-Mu insertions in pMK16 were then assayed in Type 1 reactions. As shown in Fig. 5B, all the Type 1-deficient mini-Mu's regained their ability to react at wild type levels when inserted into pMK16. These results indicated that the five different integration sites in pUC19 were responsible for reducing the efficiency of the Type 1 reaction, rather than any mutations that might have been acquired in the Mu sequences.

Fig. 4. Schematic showing the location and orientation of Type 1-deficient mini-Mu insertions in pUC19. Locations were determined by DNA sequence analysis. The number under each insertion indicates the exact integration site on pUC19. The orientation of the Mu DNA (from left to right) is indicated by an arrow.

Fig. 5. Comparison of Type 1 reaction efficiency of five enriched Type 1-deficient mini-Mu simple insertion DNA. A, the Type 1 reaction was performed as described under “Experimental Procedures,” using the initial unenriched simple insertion library DNA as a positive control (Wt). Duplicate reactions for each enriched DNA were loaded on 1% agarose gels in the absence and presence of SDS, from which the percent of Type 1 complex and nicked DNA was determined, respectively. The extent of complex formation and DNA nicking were determined by densitometry. B, comparison of Type 1 reaction efficiency of the five mini-Mu insertions in part A after transfer into pMK16. The insertion of Mu sequences into pMK16 was performed as described under the “Experimental Procedures.” The numbers in parentheses indicate the mini-Mu insertion tested in panel A and subsequently transferred into pMK16.

Fig. 6. The effect of base pair changes outside the Mu ends on Type 1 reaction efficiency. Constructs in which the Mu sequences remained intact but the flanking host sequences close to the Mu ends were changed were assayed for their ability to serve as substrates in the Type 1 reaction. pBL01 is one of the parent plasmids serving as a wild type control.
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Fig. 7. Summary of flanking host sequences in Type 1-deficient mini-Mu molecules. Flanking sequences were determined by DNA sequencing as described under "Experimental Procedures." The terminal 3 nucleotides at the left and right Mu ends are shown in the center. Five base pair duplications at the insertion site are indicated in boldface and underlined. The nucleotides in the boxed area indicate insertions outside the Mu ends, as compared with the parent plasmid pGG215. The dashed lines indicate the position of deleted nucleotides, as compared with parent plasmids pBL01 or pBL02. The plasmids are listed in order of decreasing reactivity from top to bottom in each panel. Wt, wild type.

sequences near the Mu-host junction resulted in a dramatic decrease in Type 1 reactivity.

It is noteworthy that these changes in the flanking sequences resulted in mini-Mu's without the normal direct repeats of five base pairs. Two of the parent plasmids (pBL01 and pBL02), however, had these repeats removed during their construction and displayed normal reaction efficiencies. The removal of the five base pair repeats themselves has been shown not to affect transposition in vivo (Kamp and Kahmann, 1980) or Type 1 reaction efficiency in vitro. Our results to this point indicate that the flanking host sequences close to the ends of a mini-Mu insertion can have a dramatic effect on Type 1 reaction efficiency.

Flanking Host Sequences Inhibit the Cleavage Reaction but Not Synapsis—To further define the inhibitory role of the flanking host sequences, we took advantage of the recent finding that the Type 1 reaction can be divided into two discrete steps: synapsis and cleavage (Baker et al., 1991). As shown in Fig. 8A, replacement of Mg++ with Ca++ in a Type 1 reaction results in the accumulation of an uncut, stable synaptic complex (Baker et al., 1991), which we refer to as a Type 0 complex. The presence of this complex cannot be assayed directly because it comigrates with the supercoiled plasmid in agarose gels. The presence of a Type 0 complex can be monitored, however, by restriction endonuclease cleavage, which results in the formation of a structure (Surette et al., 1991). Following the generation of a Type 0 complex, it can be rapidly converted to a Type 1 complex by incubation for 3 min in the presence of 10 mM Mg++. Fig. 8B shows that the ability of five Type 1-deficient mini-Mu's to form Type 0 complexes was not significantly compromised relative to positive controls. In contrast, all five mini-Mu's were severely affected in their ability to be converted from Type 0 to Type 1 complexes by addition of Mg++. The experiment was also repeated with the insertion and deletion mini-Mu constructs (see Fig. 7), and the same results were obtained (data not shown). The flanking host sequences in Type 1-deficient mini-Mu's are therefore inhibitory in the strand cleavage reaction but not in synapsis.

Inhibition of Cleavage by Flanking Sequences Can Be Overcome by Mu B and ATP—We have previously shown that the Mu B protein in the presence of ATP can stimulate the A protein-mediated cleavage of a mini-Mu carrying a terminal base pair mutation (Surette et al., 1991). The B protein also stimulates the intramolecular strand transfer reaction (Baker et al., 1991; Surette and Chaconas, 1991). We were therefore interested in assessing the ability of Mu B and ATP to
overcome the inhibitory effect of the flanking host sequences on the strand cleavage reaction. Fig. 9 shows the results of such an experiment. Mu B protein in the presence of ATP indeed stimulated the strand cleavage reaction with the five mini-Mu's tested. These five mini-Mu's (data not shown), as well as pMS213, 214, and 230, were also found to produce normal levels of Type 2 complex in the presence of B protein and ATP.²

DISCUSSION

It has been well established that the termini of transposable elements contain the reactive sequences required in cis for the transposition process. The work described above now establishes that host sequences that closely flank the Mu ends can have a dramatic effect on the Mu DNA strand cleavage reaction. The effect of the flanking host sequences is manifested at the cleavage step rather than synopsis, and the defect can be completely overcome by the Mu B protein in the presence of ATP. Analysis of the flanking host DNA sequences in the Type 1-deficient clones (Fig. 7) has not revealed any particular inhibitory consensus sequence. Most (but not all) Type 1-deficient clones do, however, show a very high preponderance of G-C base pairs within 5 base pairs of the nick site. G-C richness alone is not sufficient to explain the inhibitory effect on cleavage, since our wild type mini-Mu (pPG215) contains only G-C base pairs within 5 nucleotides of the nick but forms Type 1 complex at high efficiency.

It has been generally assumed that the host DNA flanking a transposon plays no role in the mobilization of a transposon. In the case of Mu DNA transposition, the emerging picture, however, is quite to the contrary. In addition to the findings reported here, recent studies on the Type 1 complex have shown that 10–13 base pairs of flanking host DNA are protected by the Mu A protein in the complex, which indicates an interaction between Mu A and flanking host sequences (Mizuuchi et al., 1991; Lavoie et al., 1991). Furthermore, in the Type 1 complex, an altered DNA structure 2 base pairs beyond the Mu-host junction on the uncut DNA strand is the flanking host sequences has been detected by enhanced hydroxyl radical reactivity (Lavoie et al., 1991). These findings allow us to speculate on two possible mechanisms by which certain flanking host sequences can inhibit the strand cleavage reaction:

1) In the first scenario, certain flanking host sequences would decrease the affinity of the Mu A protein for host DNA beyond the Mu-host junction. Decreasing the strength of this interaction might be sufficient to block cleavage.

2) The second possibility predicts that the sequence of the flanking host DNA limits its ability to exist in an altered structural state. We have recently reported that an altered DNA structure does in fact exist at the Mu-host junction in the Type 1 complex (Lavoie et al., 1991). In particular, if structural perturbations such as bending and changes in twist are required in the host sequences before nicking can occur, then DNA sequences that impede these processes would inhibit the cleavage reaction. Recent reports do suggest that both DNA flexure (Gartenberg and Crothers, 1988; Travers, 1989) and torsional rigidity (Fujimoto and Schurr, 1990) vary with base sequence; however, no predictive rules currently exist.

The data presented here do not allow us to distinguish between these possibilities, although we favor the second model. The ability of the Mu B protein to overcome the defect in the cleavage reaction is consistent with either scenario; direct interaction with Mu B could conceivably alter the binding affinity of A for flanking host DNA or help the A protein to overcome energetic barriers imposed by DNA rigidity. The role of Mu B in surfacing the cleavage defect imposed by certain flanking host sequences adds yet another important function to the growing list of stimulatory properties of this protein in the Mu DNA strand transfer reaction (see Surette et al., 1991), Baker et al. (1991), Surette and Chaconas (1991)). Since the B protein is a normal component of the Mu transposition machinery, one of its natural roles may be to ensure that efficient transposition can occur from the very large number of possible insertion sites in the host chromosome. The ability of Mu B to rescue Mu DNA from "dead-end" insertion sites is expected to be particularly important upon prophage induction or after integration of infecting Mu DNA when there is only a single copy of Mu present in the chromosome.

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