

Replacement of the Bacteriophage Mu Strong Gyrase Site and Effect on Mu DNA Replication

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The bacteriophage Mu strong gyrase site (SGS) is required for efficient replicative transposition and functions by promoting the synapsis of prophage termini. To look for other sites which could substitute for the SGS in promoting Mu replication, we have replaced the SGS in the middle of the Mu genome with fragments of DNA from various sources. A central fragment from the transposing virus D108 allowed efficient Mu replication and was shown to contain a strong gyrase site. However, neither the strong gyrase site from the plasmid pSC101 nor the major gyrase site from pBR322 could promote efficient Mu replication, even though the pSC101 site is a stronger gyrase site than the Mu SGS as assayed by cleavage in the presence of gyrase and the quinolone enoxacin. To look for SGS-like sites in the *Escherichia coli* chromosome which might be involved in organizing nucleoid structure, fragments of *E. coli* chromosomal DNA were substituted for the SGS: first, repeat sequences associated with gyrase binding (bacterial interspersed mosaic elements), and, second, random fragments of the entire chromosome. No fragments were found that could replace the SGS in promoting efficient Mu replication. These results demonstrate that the gyrase sites from the transposing phages possess unusual properties and emphasize the need to determine the basis of these properties.

Bacteriophage Mu is one of the largest and most efficient bacterial transposons known. Its 37-kb linear genome is approximately 1% the size of the *Escherichia coli* chromosome, and during the lytic cycle, approximately 100 replicative transposition events can occur within 40 min (for a general review, see references 10 and 21).

Studies with Mu have been instrumental to our understanding of the mechanism of transposition. Analysis of transposition in an in vitro system with purified components has led to a detailed description of the steps in the transposition pathway (reviewed in references 15 and 17). The in vitro system, however, uses as a substrate a small plasmid containing short segments of the Mu DNA termini to approximate the 37-kb genome which normally undergoes replicative transposition within the bacterial nucleoid. Hence, some aspects of Mu biology may not be addressed by the in vitro system.

We have been interested in synapsis of the prophage termini by transposase, an obligate early step in the transposition pathway, which, in vivo, involves long-range DNA interactions within the constraints imposed by the structure of the nucleoid. Transposase monomers bind to three sites at each end of the prophage and to an enhancer region near the left end (35). Following synapsis of the prophage ends, the transposase monomers rearrange to form a stable tetramer bound to two sites at the right end and one site at the left end. Synapsis is essential for transposition, because transposase acts in *trans* to generate the required cleavage and ligation reactions (i.e., transposase bound at the left end cleaves at the DNA junction at the right end and vice versa) (1, 27, 37). We have proposed that a site located in the center of the prophage promotes synapsis of the termini and that it does so by organizing the structure of the prophage DNA into a plectonemically interwound supercoiled loop with the site at the apex of the loop

and the termini to be synapsed at the base (24). We found a site in the center of the genome that is required for efficient Mu replicative transposition and identified it as a strong DNA gyrase cleavage site (SGS) (24). Deletion of the SGS apparently inhibits transposition at the step of synapsis of the prophage termini (25). Several experimental approaches were used to demonstrate that the SGS has to be symmetrically located between the termini to be synapsed to allow for optimal rates of Mu replication (22, 23).

If the Mu SGS is capable of organizing the topology of prophage DNA as suggested by the model, then an important question that arises is whether this use of a strong gyrase site is unique to the Mu site or whether it is a more general property of a class of sites. For example, the *E. coli* nucleoid is thought to be composed of 50 to 100 independently supercoiled domains (13, 30, 36), and one could ask whether gyrase sites might be involved in the formation of individual domains. Detection of gyrase sites in the *E. coli* chromosome that are preferentially cleaved in the presence of a quinolone (4, 31), possibly associated with certain repetitive DNA elements (7, 38), has been cited to support the notion of the involvement of gyrase sites in nucleoid structure. To approach the question, we have chosen to search for sites which could replace the Mu SGS in allowing efficient replication of Mu DNA. The approach used was to clone fragments of DNA from various sources into the center of a prophage lacking the SGS and to examine the effects of the new site on Mu replication.

MATERIALS AND METHODS

Standard cloning procedures were used throughout. Ligations of restriction fragments with incompatible ends were done after end filling with Klenow polymerase or T4 polymerase. The coordinates given for restriction sites in Mu DNA are in kilobases from the left end of the 37.2-kb genome.

Construction of the Δ 100 plasmid pMP1856. In a previous publication (23), we described the construction of the plasmid pMP1812, which contains (i) the *Bam*HI-*Eco*RI (17.2 to 23.7 kb) fragment of Mu DNA cloned at the *Bam*HI site of a pBR322 plasmid with deletion of its ampicillin resistance (*Ap^r*) gene; (ii) a 147-bp *Mlu*I-*Sca*I (18.0 to 18.15 kb) deletion removing the SGS and the 3' end of the upstream G gene; (iii) an inserted oligonucleotide restoring the end of the G gene and introducing sites for *Bgl*II, *Bst*II, and *Stu*I; and (iv) a *Kn^r* cassette at

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TABLE 1. Bacterial strains, plasmids, phages, and lysogens used in this study

Phage, plasmid, strain, or lysogen	Characteristics
Phages	
Mu cts62	
D108 cts10	
Plasmids	
pMP1812.....	pBR322 Δ (3.2–4.3 kb); + Mu <i>Bam</i> HI- <i>Eco</i> RI (17.2–23.7 kb) at <i>Bam</i> HI site; Δ 100 (Mu Δ 18.05–18.15 kb); <i>Kn</i> ^r cassette at site of Δ 100 deletion (see reference 19 for details).
pMP1852.....	As pMP1812, except <i>Ap</i> ^r cassette at site of Δ 100 deletion
pMP1856.....	As pMP1812, except <i>Ap</i> ^r cassette at <i>Bam</i> HI site at Mu-pBR322 junction.
Bacterial strains	
AB1157	
DH5 α	
Lysogens	
X20.....	AB1157 <i>recB recC sbcB malF::Mu cts62</i>
MP1928.....	X20 Δ 100 (Mu Δ 18.05–18.15 kb) + <i>Sac</i> Kn insert
MP1998.....	MP1928 – <i>Sac</i> Kn insert
MP2013.....	MP1928 – <i>Sac</i> Kn insert + Mu SGS insert (+ orientation)
MP2015.....	MP1928 – <i>Sac</i> Kn insert + Mu SGS insert (– orientation)
MP2014.....	MP1928 – <i>Sac</i> Kn insert + D108 insert
MP1983.....	MP1928 – <i>Sac</i> Kn insert + pSC101 insert
MP1987.....	MP1928 – <i>Sac</i> Kn insert + pBR322 insert
MP1996.....	MP1928 – <i>Sac</i> Kn insert + <i>nrd</i> BIME insert
MP2001.....	MP1928 – <i>Sac</i> Kn insert + <i>asl</i> BIME insert
MP2033.....	X20 Δ 200 (Mu Δ 17.95–18.15 kb) + <i>Kn</i> insert

the *Stu*I site. To construct pMP1856, the *Kn*^r cassette was deleted from p1812 and an *Ap*^r cassette was introduced at the *Bam*HI junction of Mu and plasmid DNA, resulting in a net deletion of about 100 bp from 18.05 to 18.15 kb (Table 1).

Construction of Δ 100 and Δ 200 prophages. A 3.5-kb DNA fragment containing the *sacBR* genes and a *Kn*^r cassette, derived from pSUP104-sac (29), was cloned into the *Bgl*II site in pMP1856. The resulting plasmid was linearized and transformed into strain X20 (W3110 *recB recC sbcB malF::Mu cts62*). A *Kn*^r recombinant was selected which had recombined the Δ 100 deletion and the *sac* *Kn*^r fragment into the resident prophage to generate strain MP1928. Then to construct prophages containing DNA fragments to be tested for their ability to replace the SGS, the fragments were cloned into the *Bgl*II site on pMP1856, and the resulting plasmids were linearized and transformed into MP1928. Recombinants which had replaced the *sac* *Kn*^r fragment in the prophage with the desired DNA fragment were selected for sucrose resistance on L-agar plates with no salt and with 5% sucrose and screened for kanamycin sensitivity, and the substitution with the desired fragment was verified by restriction analysis and by PCR.

To construct a prophage that lacks the SGS and is G[–] (the original 147-bp deletion removed the terminal seven amino acids of G and was slightly leaky), a larger deletion was created (*Bgl*I-*Sca*I; bp 17.95 to 18.15) which removed approximately 200 bp; this prophage is referred to as the Δ 200 prophage and is in the lysogen MP2033.

Construction of chromosomal libraries in pMP1856. Total *E. coli* chromosomal DNA was isolated by phenol extraction and fragmented by complete or partial cleavage with *Sau*3A or partial digestion with DNase I in the presence of 10 mM MnCl₂. The DNA was size fractionated on 1.2% agarose gels, and fragments in the range of 0.5 to 1.5 kb were isolated (use of larger inserts could have resulted in loss of the right end of the genomes containing the inserts due to the “headful” packaging mechanism of Mu DNA). *Sau*3A-generated fragments were cloned directly into the *Bgl*II site of pMP1856 after calf intestinal phosphatase treatment of the digested plasmid DNA. To assess the efficiency of the cloning procedure, plasmid DNAs from 10 independent *Ap*^r transformants were isolated and checked for insertions; greater than 80% contained insertions of the expected sizes. The ends of DNase I-generated fragments were filled in with T4 DNA polymerase, *Bam*HI linkers were added, and the fragments were cloned into the *Bgl*II site of pMP1856.

Growth, lysis, and replication. Cultures of lysogens were grown in L broth at 30°C to a density of about 10⁸ cells/ml, diluted threefold with L broth, and induced by transferring the culture to 42°C. In the absence of dilution, some cultures reached densities which were sufficiently high to delay lysis beyond the

times seen at lower densities. Culture density was determined with Klett readings to monitor growth and lysis.

Replication was monitored by a semiquantitative PCR procedure. Two hundred microliters of induced cultures was added to tubes with 0.04 g of Chelex and 2 μ l of 0.1 M EDTA and boiled for 10 min. Ten microliters or less of the extracts (the volume of extract used was corrected for the amount of growth during the sampling regime) was used in PCRs designed to yield results in a linear range. Pairs of PCR primers were used to simultaneously amplify a fragment of Mu DNA and a fragment of chromosomal DNA (from a gene adjacent to the prophage at *malF*). The Mu primers 5'-TGATGAGGGTACACTTGCTGG and 5'-GCACAGATGCTGTAATGGTTCG yield a 335-bp product from the Mu B gene. The chromosomal primers 5'-TTAAGCCATCTCTGATGACG and 5'-TTTCTGCTACTGACAGGTGGG yield a 364-bp fragment produced from the *malK* gene. Following 20 cycles of amplification, the amplified DNA fragments were separated on 2% agarose gels, stained with ethidium bromide, and quantitated with NIH Image software v. 155. Control experiments performed by mixing different ratios of the two products showed that linearity was observed up to a ratio of about 5 to 1. The ratio of Mu to *mal* is close to 1.0 in uninduced cells and increases after the onset of Mu DNA replication.

Gyrase cleavage in vitro and in vivo. In vitro gyrase cleavage assays were carried out as described by Pato et al. (24) on plasmid DNA linearized with *Pvu*II digestion. In vivo cleavage assays were modified from the method of Scheirer and Higgins (28). A relevant lysogen was grown in L broth at 30°C to a Klett density of 50, and enoxacin was added to 300 μ g/ml. After 5 min of incubation, 5 ml of culture was rapidly lysed by transfer to a tube at 80°C containing 0.5 ml of L broth plus 2.5% sodium dodecyl sulfate (SDS), and incubation was continued for 15 min. The sample was cooled to room temperature, proteinase K was added to 20 μ g/ml, and incubation was continued for 1 h at 65°C. DNA was purified by phenol-chloroform extractions and ethanol precipitation and was digested with the appropriate restriction enzyme. A single oligonucleotide primer, complementary to a sequence chosen so that the gyrase site is between the primer site and the restriction site, was 5' end labeled with ³²P and used for 30 cycles of one-directional PCR. The extension products terminated either at the cleaved gyrase site (for templates cleaved by gyrase in vivo) or at the cleaved restriction site (for templates not cleaved by gyrase). The resulting labeled fragments were separated on a sequencing gel and quantitated with a phosphorimager.

RESULTS

Replacing the SGS. To replace the SGS in the center of a Mu prophage with DNA fragments from various sources, two procedures were developed—one to insert specific, known fragments and the other to insert random DNA fragments from cloned libraries.

In the first procedure, the DNA fragments to be tested were cloned into a central fragment of Mu DNA from which the SGS was deleted and then recombined into a prophage inserted at a unique site in the *E. coli* chromosome. For this procedure, a plasmid was constructed which carries a central fragment of the Mu genome from *Bam*HI to *Eco*RI (17.2 to 23.7 kb). Approximately 100 bp was deleted from the fragment, removing the SGS, and replaced with an oligonucleotide introducing a *Bgl*II site (pMP1856; see Materials and Methods). Selected DNA fragments were then cloned into the *Bgl*II site. The plasmid was linearized and transformed into a *recB recC sbcB* lysogen carrying a Mu prophage with the Δ 100 deletion and a 3.5-kb fragment containing the genes for sucrose sensitivity and kanamycin resistance (*sac* *Kn*^r) at the site of the deletion (MP1928). A recombinant was then selected which replaced the *sac* *Kn*^r genes with the fragment carried on the plasmid. The structure of the resulting recombinant was verified by PCR analysis and, when desired, sequencing. The resulting prophages are all at the same chromosomal site (*malF*) and do not contain drug-resistant gene cassettes (which were present in some earlier constructs).

To test the system, a 147-bp fragment containing the Mu SGS (*Mlu*I-*Sca*I; 18.0 to 18.15 kb) was cloned into the *Bgl*II site of pMP1856 in both orientations and recombined into the prophage in MP1928. Also, pMP1856 without an insert was used, resulting in a prophage with only the Δ 100 deletion. The lysogens were grown at 30°C and then induced by shifting to 42°C, and growth of the culture and Mu DNA replication were monitored (Fig. 1). The lysis times for lysogens with the Mu

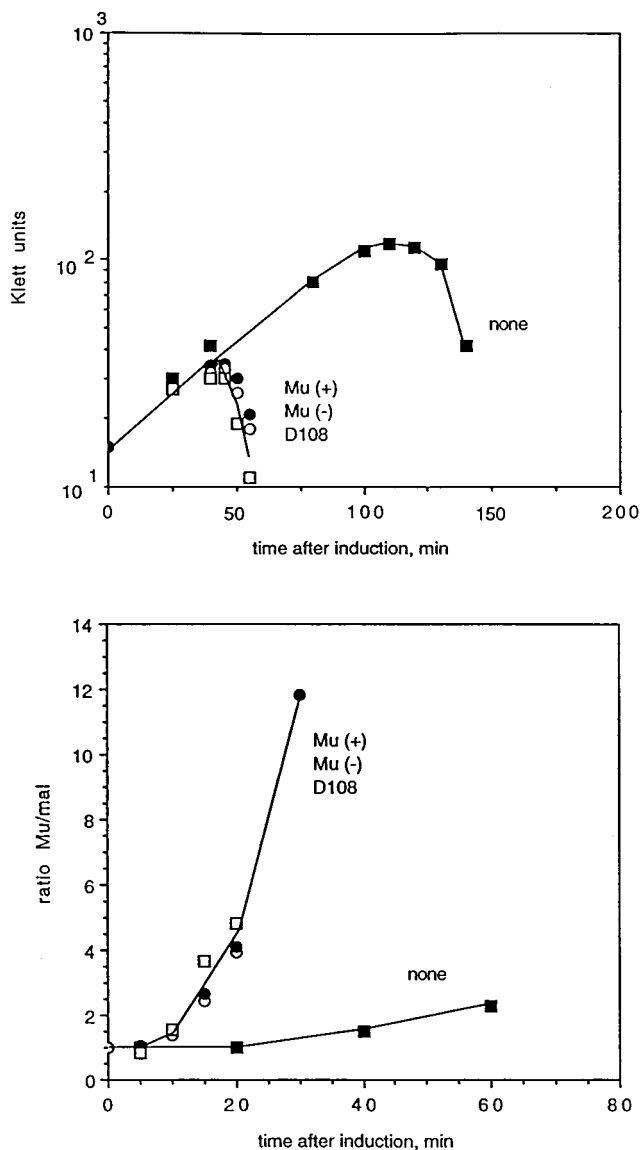


FIG. 1. Lysis and replication without a central gyrase site (■), with the Mu SGS in the positive (○) or negative (●) orientation, or with the D108 gyrase site (□). Cultures of lysogens were grown in L broth at 30°C to a density of about 10⁸ cells/ml, diluted threefold in L broth, and then induced by shifting to 42°C. Growth was monitored by Klett readings, and samples were taken for quantitative PCR analysis to measure DNA replication. Replication is expressed as the ratio of the amount of an amplified Mu DNA fragment to the amount of a chromosomal *malF* fragment. (Top panel) Growth and lysis of the cultures. (Bottom panel) Mu DNA replication.

inserts in either orientation were essentially identical to that for wild-type Mu, as were the kinetics of Mu DNA replication. The lysogen carrying a prophage lacking the SGS showed long delays before lysis and the onset of Mu DNA replication. A strict correlation between the kinetics of lysis and Mu DNA replication has been observed in all of our work with modified Mu prophages (with obvious exceptions, such as prophages deleted for the *lys* genes). For rapid screening of many prophage constructions, lysis curves alone were used, because they have the advantage of ease of performance and less experimental fluctuation.

Bacteriophage D108. The DNA of the transposing bacteriophage D108, a close relative of Mu (6), was examined, because

it seemed likely that D108 would have a functional central gyrase site. Initially, a 1.4-kb central fragment of D108 (*Bam*HI-*Cl*AI; corresponding to Mu 17.2 to 18.4 kb) was cloned into pBR322, and about 250 bp of sequence were obtained in the region corresponding to the Mu SGS. The sequence (Fig. 2) was identical to that of Mu, except for 4 base changes, 3 of which were in or near the presumed gyrase site. The *Mlu*I and *Sca*I sites used for cloning the Mu SGS are also present in D108, and hence the corresponding 147-bp fragment from D108 was cloned into pMP1856 and recombined into the Mu prophage in MP1928. Induction of the lysogen with the hybrid prophage gave essentially the same results as those obtained with wild-type Mu (Fig. 1), showing that the Mu SGS could be successfully replaced with the corresponding site from D108.

To determine if the D108 site is indeed a gyrase site, cleavage of the D108 DNA in the presence of gyrase and the quinolone enoxacin was examined. Quinolones inhibit the gyrase reaction after the enzyme creates a double-strand break in the bound DNA, and the protein is covalently linked to the cleaved DNA ends (9, 33). Hence treatment with gyrase and a quinolone followed by deproteination can reveal a double-strand break. For the analysis, the 1.4-kb central fragments from Mu and D108 were cloned into pBR322. The plasmids were linearized by restriction enzyme digestion and cleaved with gyrase in the presence of enoxacin (see Materials and Methods). After treatment with SDS and proteinase K, the resulting fragments were separated on an agarose gel, stained with ethidium bromide, and photographed. The results in Fig. 3 show that gyrase cleavage at the D108 site occurred with approximately the efficiency observed at the Mu site.

To determine the effect on D108 of deleting the gyrase site, the *Mlu*I-*Sca*I fragment was deleted from the 1.4-kb central fragment described above and replaced with a 1.1-kb *Kn*^r cassette, and the deletion was recombined into a D108 *cts* prophage. Induction of the resulting lysogen resulted in delays in lysis and D108 replication equivalent to those seen with the corresponding Mu ΔSGS prophage (data not shown).

pSC101 and pBR322 gyrase sites. We wished to examine the effect of replacing the Mu SGS with known gyrase sites and began by selecting two well-studied sites from the plasmids pBR322 and pSC101. A large number of gyrase sites on pBR322 have been described (16), and the major site centered on base 991 has been used as substrate in much of the work with gyrase (e.g., see references 5 and 19). A strong gyrase site has been observed on pSC101, where it was originally identified as part of the *par* region of the plasmid (34). A 775-bp

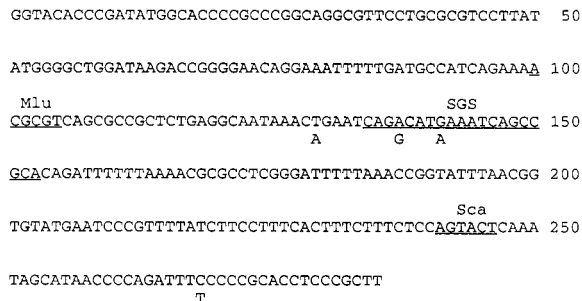


FIG. 2. Sequence of a region of the D108 genome containing the central strong gyrase site. Mismatches with the Mu sequence are noted, and the locations of the gyrase site consensus sequence (denoted as SGS) and the restriction sites for *Mlu*I and *Sca*I are underlined. (The GenBank accession no. is Bank It 253889 AF 128885.)

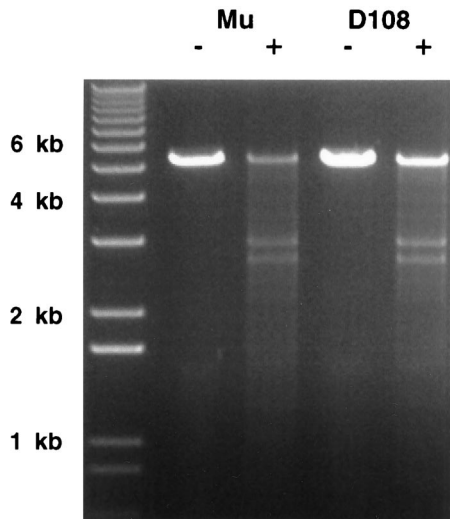


FIG. 3. Gyrase cleavage of Mu and D108 DNA. pBR322 Δ (*EcoRI-BamHI*) plasmids with cloned 1.4-kb central fragments from Mu (lanes 2 and 3) and D108 (lanes 4 and 5) were linearized by *PvuII* restriction digestion and then incubated with DNA gyrase in the presence of enoxacin. Following treatment with SDS and proteinase K, the DNA fragments were separated on a 1% agarose gel and stained with ethidium bromide. The expected fragment sizes for cleavage at the Mu gyrase site are \sim 2.9 and 2.6 kb. Lane 1, molecular size markers. -, no gyrase; +, with gyrase.

fragment of pBR322 and a 400-bp fragment of pSC101 carrying the gyrase sites were separately cloned into pMP1856 and recombined into the prophage in MP1928. Induction curves for the resulting lysogens show that neither of the fragments was capable of restoring normal replication (Fig. 4). However, the pSC101 fragment did have a measurable effect, reducing the lysis time from 110 min to about 90 min and allowing some increase in the rate of Mu replication over that seen in the absence of a gyrase site.

The inability of the plasmid gyrase sites to replace the Mu SGS in promoting efficient Mu replication could be due to their being weaker sites than the SGS. To assess the relative strengths of the three gyrase sites, we performed both in vitro and in vivo cleavage assays with the quinolone enoxacin. For the in vitro analysis, small fragments with the different gyrase sites were cloned into pBR322. The plasmids were linearized by restriction enzyme digestion, cleaved with gyrase in the presence of enoxacin, and processed as described above. The first three pairs of lanes in Fig. 5 show results with pBR322, without and with the Mu and pSC101 gyrase sites. Even within the context of the greater than 40 weak gyrase sites in pBR322, specific cleavages were readily observed at the Mu and pSC101 gyrase sites. The pSC101 site was cleaved more efficiently than the Mu site, and under the conditions used, no clear cleavage at the major pBR322 site was observed.

For the in vivo analysis, lysogens with different central gyrase sites were used. Exponentially growing cultures were treated with enoxacin for 5 min, followed by rapid lysis in SDS at 80°C. DNA was isolated, deproteinized with proteinase K, and cleaved with a selected restriction enzyme. One-directional PCR using a primer near each of the gyrase cleavage sites was performed. If gyrase cleavage had occurred on a template molecule, primer extension could proceed up to the gyrase site; if gyrase cleavage had not occurred, extension could proceed past the gyrase site up to the restriction site, yielding a longer fragment. The ratios of the amount of the shorter fragment to the total of the two fragments were determined and were \sim 40% with the Mu gyrase site and \sim 60% with the pSC101

gyrase site (Fig. 6). No clear gyrase cleavage was observed with the pBR322 site, consistent with the lack of observable cleavage in the in vitro assay. By this assay, the pSC101 gyrase site is even stronger than the Mu SGS, and the pBR322 site is very weak relative to the other two. Because the pSC101 gyrase site was unable to efficiently promote Mu replication (Fig. 4), it appears that replacing the function of the Mu SGS in Mu replication requires more than just the introduction of a strong gyrase site.

The *E. coli* chromosome—BIME sites. The *E. coli* nucleoid is thought to be composed of 50 to 100 independently supercoiled domains (30, 36). Since the Mu SGS can promote the organization of the Mu prophage DNA into a supercoiled domain, one wonders if there are chromosomal sites which could promote domain formation in an analogous manner. To

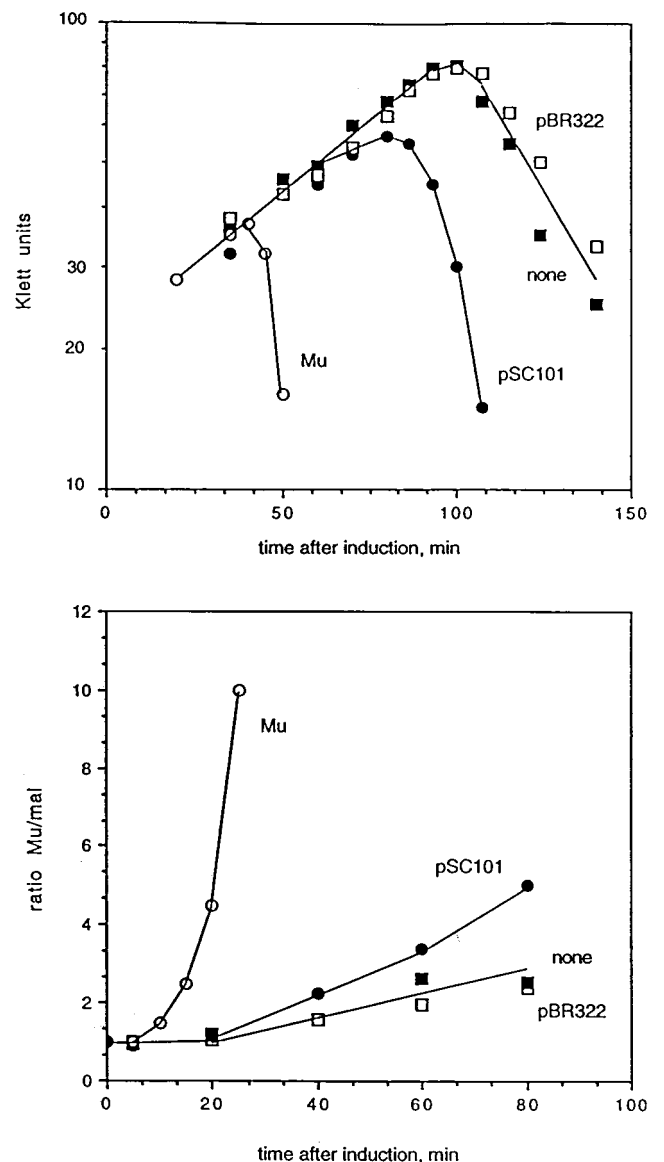


FIG. 4. Lysis and replication without a central gyrase site (■), with the Mu SGS in the positive orientation (○), with the pSC101 gyrase site (●), or with the pBR322 gyrase site (□). Growth and replication were monitored as in Fig. 1. (Top panel) Growth and lysis of the cultures. (Bottom panel) Mu DNA replication.

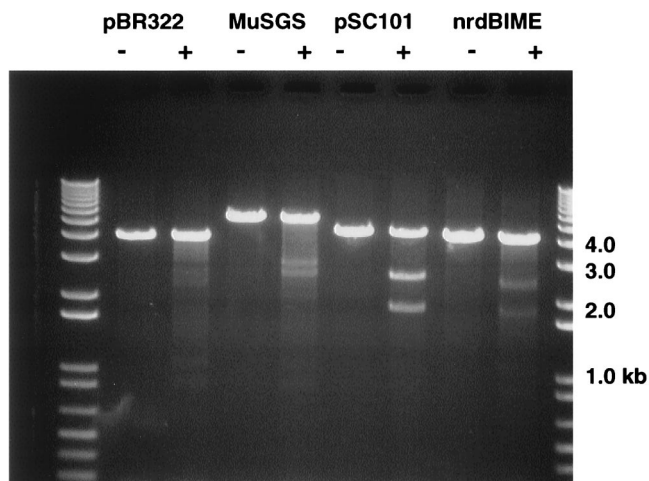


FIG. 5. In vitro gyrase cleavage. pBR322 Δ (*EcoRI*-*Bam*HI) plasmids without (lanes 2 and 3) or with cloned fragments carrying the Mu SGS (lanes 4 and 5), the pSC101 gyrase site (lanes 6 and 7), or the *nrdAB* BIME (lanes 8 and 9) were linearized with *Pvu*II restriction digestion and then incubated with DNA gyrase in the presence of enoxacin. Following treatment with SDS and proteinase K, the DNA fragments were separated on a 1% agarose gel and stained with ethidium bromide. The expected fragment sizes for cleavage at the gyrase site in question were as follows: pBR322, 2.8 + 1.2 kb; Mu, 2.9 + 2.6 kb; pSC101, 2.5 + 1.9 kb; *nrdAB* BIME, 2.4 + 1.8 kb. Lanes 1 and 10, molecular size markers. -, no gyrase; +, with gyrase.

seek chromosomal sites capable of replacing the Mu SGS, we have used two approaches. The first involved replacing the SGS with known chromosomal sites called bacterial interspersed mosaic elements (BIMEs). These elements are composed of combinations of repetitive extragenic palindrome (REP) sequences, which are scattered throughout the genome, are often associated with gyrase binding, and have been proposed as organizing elements for the nucleoid (reviewed in reference 2). Two classes of BIMEs have been defined: BIME-1 sites contain integration host factor (IHF) binding sites flanked by REPs; BIME-2 sites contain a particular pattern of REPs without an IHF binding site. We chose to examine the *nrdAB* BIME-2, because it contains the strongest gyrase site of the several sites examined by Espeli and Boccard (7), and the *aslAB* BIME-1. A 250-bp fragment containing the *nrdAB* BIME-2 and a 240-bp fragment containing the *aslAB* BIME-1 (generous gifts from F. Boccard) were separately cloned into pMP1856 and recombined into the Mu prophage in MP1928. Induction of the resulting lysogens showed that neither of the BIMEs was capable of replacing the Mu SGS in supporting efficient Mu replication, because the lysis times were essentially identical with and without the BIME insertions (data not shown).

The *nrdAB* BIME contained the strongest gyrase site of several BIMEs examined by Espeli and Boccard (7). In vitro gyrase cleavage was performed with pBR322 with a cloned *nrdAB* BIME insert and showed a degree of cleavage similar to that observed with the plasmid containing the Mu SGS insert (Fig. 5). Interestingly, no preferential cleavage was observed with the in vivo gyrase cleavage assay (data not shown).

The *E. coli* chromosome—random fragments. The second procedure used to search for chromosomal sites capable of replacing the Mu SGS was to construct libraries of total *E. coli* DNA cloned into the *Bgl*II site in the Δ 100 plasmid pMP1856 and then to recombine the random fragments into the center of a Mu genome lacking the SGS. For these experiments, strain MP2033 was constructed, which carries a Mu prophage with a

deletion of approximately 200 bp, removing both the SGS and the 3' end of the upstream G gene. The G gene product is required for proper assembly of phage heads and tails (12), and G⁻ phage lysates do not produce plaque-forming particles. In contrast, phage with the Δ 100 deletion which lack the SGS but are G⁺ are capable of forming pinpoint plaques on some hosts (*E. coli* DH5 α) while incapable of forming plaques on other hosts (*E. coli* AB1157).

The basis of the selection is the following: recombination between the Δ 200 genome and a Δ 100 fragment on a plasmid yields a Δ 100 genome which can at least give rise to a pinpoint plaque on DH5 α . If an *E. coli* fragment capable of substituting

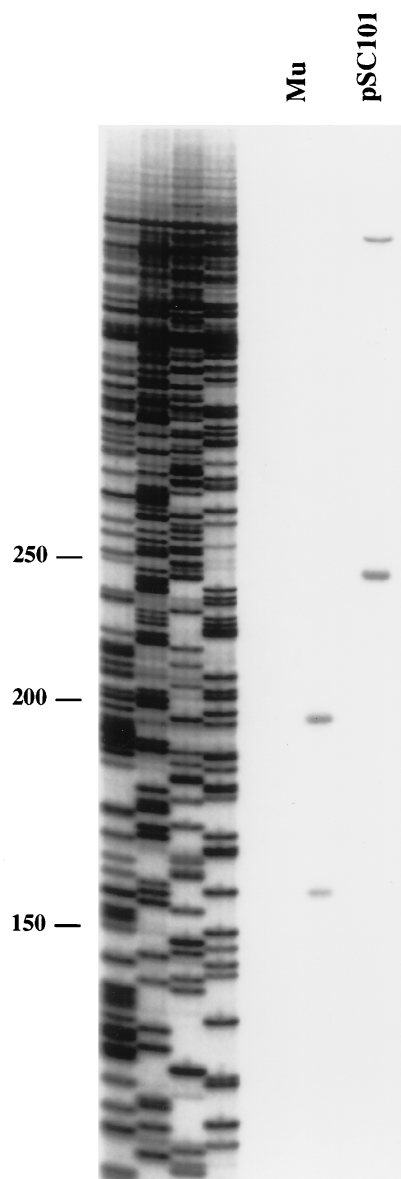


FIG. 6. In vivo gyrase cleavage. Lysogens with prophages carrying either the Mu SGS or the pSC101 gyrase site were grown in L broth, enoxacin was added to 300 μ g/ml for 5 min, and the cells were rapidly lysed in hot SDS. DNA was isolated, treated with proteinase K, and digested with the appropriate restriction enzyme. One-directional PCR with primers near the gyrase sites yielded shorter fragments from templates that were cleaved by gyrase and longer fragments from templates not cleaved by gyrase. A sequencing ladder provided size markers (base pairs).

for the Mu SGS has been recombined into the center of the phage genome, then the recombinant phage could form a plaque on an AB1157 host. Hence, the plasmid libraries were transformed into the Mu $\Delta 200$ lysogen, large numbers of individual transformants were pooled, and the pooled culture was induced for Mu growth. Lysates collected several hours after induction were plated on the appropriate indicator strains, and plaques were counted.

To test the system, a size-fractionated library of Mu DNA fragments, approximately 1 kb in size, was cloned in pMP1856 and electroporated into the $\Delta 200$ lysogen. Colonies of plasmid-containing (ampicillin-resistant) transformants were scrapped from plates and pooled. Cultures of the pooled cells were induced, and lysates collected after ~ 3 h were plated in top agar on the following different indicators: a, DH5 α , to determine the number of G⁺ recombinants; b, DH5 α carrying a plasmid supplying G protein in *trans* (pMP1852), to determine the total number of phage in the lysate; and c, AB1157, to determine the number of recombinants carrying the SGS or SGS substitute ("Jackpots").

The numbers of pinpoint plaques on indicators a and b allowed estimates of approximately 10 phage produced per induced cell after 150 min, of which about 1/10⁴ was a G⁺ recombinant. Approximately 1% of these recombinants gave normal-size plaques on both DH5 α and AB1157. PCR and restriction digestion analyses of the DNA of these "Jackpot" phage revealed inserts corresponding to the central, SGS-containing fragment. No fragments from other portions of the genome were observed in Jackpot phage. These results demonstrated that the system could be used in a search for sites capable of replacing the SGS in Mu replication.

Libraries of *E. coli* DNA were constructed in pMP1856 by several techniques, including *Sau*3AI partial and complete digests and digestion with DNase in the presence of Mn²⁺. In numerous independent experiments, 0.5- to 1.0-kb fragments were cloned into the *Bgl*II site of the $\Delta 100$ plasmid and electroporated into the $\Delta 200$ lysogen. The plasmids from numerous independent transformants were analyzed, and most or all plasmids were found to contain inserts. Colonies of cells containing the plasmid were pooled—generally about 5,000 colonies per pool and 20,000 colonies total in each experiment. The pooled cells were grown to $\sim 10^8$ cells/ml and induced, and sufficient amounts of lysates were plated on AB1157 to contain at least 50,000 G⁺ recombinants. An experiment using *E. coli* chromosomal DNA from a Mu lysogen revealed the presence of a small number of Jackpot phage which were shown to carry the Mu SGS, but no Jackpot phage were found with *E. coli* DNA from the nonlysogen. Jackpot phage were observed with lysogen DNA at a frequency of approximately 1/10⁴ G⁺ recombinants, and greater than 10⁵ G⁺ recombinants were processed with nonlysogen DNA; hence the selection technique would likely have detected chromosomal sites capable of substituting for the SGS if they were present. The failure to find such sites emphasizes the unusual properties of the Mu SGS.

DISCUSSION

Previous studies in this laboratory have demonstrated that there is a site in the center of the Mu genome that is required for efficient replicative transposition. The site was shown to contain a strong gyrase site, which may be both necessary and sufficient to fulfill the role of the SGS in promoting Mu replication. To test whether other sites, in particular other gyrase sites, could replace the Mu SGS in promoting Mu replication, we replaced the SGS with known gyrase sites or with random fragments of the *E. coli* chromosome and evaluated the effects

on Mu replication. A central fragment from the related transposing bacteriophage D108 successfully replaced the Mu SGS in promoting Mu replication, and we demonstrated that the D108 site is indeed a strong gyrase site comparable to the Mu SGS.

The gyrase site from the plasmid pSC101 was found to be a stronger site than the Mu SGS, as measured by cleavage in the presence of gyrase and the quinolone enoxacin, both *in vitro* and *in vivo*. However, it was not able to promote efficient Mu replication, ruling out the hypothesis that any strong gyrase site can replace the Mu SGS. A slight increase in the level of Mu replication was observed with the pSC101 site over that seen in the absence of any central site. The major gyrase site from pBR322 was much weaker than the Mu or pSC101 sites in the cleavage assay and showed no ability to promote Mu replication. We found that the *E. coli* chromosome has no sites which can replace the Mu SGS in promoting efficient replication, even though sites such as the *nrdAB* BIME exhibit activity in the cleavage assay comparable to that seen with the Mu SGS.

It may be useful to review the evidence that a gyrase site is required for efficient Mu replication. (Please note that the requirement for the central site is not an absolute one; replication in the absence of the site does occur, but is delayed by approximately an hour.)

(i) A strong gyrase site is present, as defined by the quinolone cleavage assay (24). (ii) Deletion of 100 bp of noncoding sequence at the site inhibits replication (24). (iii) Insertion of a short oligonucleotide linker at the gyrase cleavage site inhibits replication (25). (iv) Replication of Mu is inhibited in gyrase mutants, and two mutants of Mu (*nuB*) which were selected for their ability to grow on a gyrase mutant (39) contain single base changes in the central gyrase site which increase the strength of the site, as measured by the quinolone cleavage assay (24). More recently isolated *nuB* mutants contain the same mutation as one of the original mutants (3). (v) Replacement of the SGS with the strong pSC101 site did allow a discernible improvement of Mu replication over that observed in the absence of any central site, although considerably less than normal replication (this work).

It is therefore likely that a central gyrase site is required for efficient Mu replication. However, the inability of strong sites such as the pSC101 or *nrdAB* BIME sites to substitute for the Mu SGS suggests that some additional property of the SGS is required.

The structures of a few gyrase sites have been probed with footprinting techniques, and a model has evolved in which about 120 to 140 bp of DNA is wrapped around the enzyme (reviewed in reference 26). A central core of DNA, estimated to be about 30 to 40 bp with DNase I (8, 14, 18) or about 13 bp with hydroxyl radicals (20), is strongly protected in the footprinting analyses and is thought to be bound at the active site of the enzyme. This core roughly corresponds to the 20-bp consensus sequence derived from a large number of weak gyrase sites on pBR322 (16) and includes the actual cleavage sites. Flanking the core are two regions, or arms, which show enhanced sensitivity in the footprinting analyses at intervals of 10 to 11 bp, suggesting that these arms are wrapped around the enzyme (8, 14, 18, 20). As with nucleosomal DNA, the arms may require inherent flexibility rather than specific DNA sequences.

Mutation and deletion analyses of the Mu SGS and the pSC101 gyrase site are in progress and should allow us to determine whether the unusual properties of the SGS are due to specific regions of the gyrase site or, for example, are due to the presence of a second site. Various possibilities can be envisioned, such as the presence of a site responsible for in-

creased processivity of gyrase action at the SGS. Completion of these studies will hopefully provide an understanding of the unusual properties of the central gyrase sites of the transposing phages.

One of the reasons for undertaking the present studies was the hope of learning something about the structure of bacterial nucleoids. Although the model for the structure of the *E. coli* nucleoid was proposed over 20 years ago, we still do not know if the structure is a static or a dynamic one, in the sense of whether the independently supercoiled loops are fixed structures or ones that are constantly in flux. Experiments such as those of Higgins and coworkers (11, 32) point toward an interpretation of a dynamic structure. The failure to find chromosomal sites which can replace the Mu SGS perhaps can best be understood in terms of a dynamic structure for the nucleoid. That is, the purpose of the Mu SGS is to promote the formation of a fixed structure—a single domain containing the Mu prophage with its termini synapsed at the base of a loop by a transposase tetramer. Analogous chromosomal sites might restrict the movement of the DNA within the nucleoid. If the nucleoid is a dynamic structure, with alternative plectonemically intertwined loops continually being formed and dissipated, then it is still possible that weaker gyrase sites are involved in initiating the formation of the alternative domainal loops.

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