Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7056–7060, July 1994
Biochemistry

Central location of the Mu strong gyrase binding site is obligatory for optimal rates of replicative transposition

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Communicated by Norman R. Pace, April 11, 1994

ABSTRACT **The bacteriophage Mu genome contains a strong DNA gyrase binding site (SGS) near its center, and disruption of the SGS by deletion or by insertion results in long delays in replication following induction of the appropriate lysogen. To determine if the central location of the SGS is obligatory for its function in Mu replication, we pursued two lines of investigation. First, fragments of Mu DNA containing the SGS were inserted into various locations in a Mu prophage lacking the central SGS. Replication following induction was restored in all of the lysogens constructed, but the observed rate of replication for different prophages decreased with increasing distance between the new location of the SGS and the center of the genome. We also deleted different lengths of DNA from within the right half of a wild-type prophage, retaining the SGS and displacing it from a central location. Replication rates of the deleted prophages were reduced, with larger deletions resulting in larger reductions. Pairing deletions in the right half of the prophage with a deletion in the left half resulted in substantially higher rates of replication than observed with the right half deletions alone. We conclude that the SGS must be located centrally between the Mu termini for optimal rates of Mu replication. These results are discussed in terms of a model that proposes that the SGS is involved in organizing the topology of supercoiled prophage DNA to assist in synapsis of the Mu termini.**

Bacteriophage Mu replicates its DNA following induction of a lysogen or infection of a sensitive host by a series of replicative transpositions. Mu is unusual, however, among transposons of *Escherichia coli* both in its large size—37.2 kb—and efficiency of transposition—replication begins within a few minutes of induction and 100 copies are produced in less than an hour (for review, see ref. 1).

Curiosity about potential problems associated with the length of the Mu genome prompted us to investigate the effects of increasing the genome length with a series of insertions, creating a group of constructs called "maxi-Mus." Dramatic decreases in rates of replication and transposition were observed with increasing lengths of insertions (2). The observed inhibition of replication was shown to occur at the level of initiation and was not the result of premature termination of replication on the lengthened prophages.

One model I considered to explain the decreased replication potential of maxi-Mus proposes that a site in the center of the Mu genome is involved in organizing the topology of supercoiled prophage DNA to assist in the synapsis of the Mu termini; the insertions in the maxi-Mus would interfere with synapsis by upsetting the symmetrical placement of the organizing site. Three predictions arise from this model: (*i*) there is a site in the center of the genome that is required for efficient Mu replication; (*ii*) in the absence of the site, replication is inhibited at the stage of synapsis of the Mu termini; and (*iii*) central location of the site is obligatory for its function in Mu replication.

To examine the first prediction, which requires a site that affects DNA topology, we sought, and found, an unusually strong gyrase binding site (SGS) near the center of the genome at 18.1 kb, between two late operons (ref. 3; Fig. 1). Deletion of 147 bp (18.0–18.15 kb) containing the site strongly inhibited replication following induction; a delay of more than an hour before commencement of replication was observed.

Experiments to test the second prediction led to characterization of prophage lacking the SGS. Results showed that the block in replication occurred at a stage in Mu development after early transcription and the synthesis of required replication proteins but before the stage at which the ends are synapsed and nicks are introduced at the Mu termini (unpublished data).

In the present communication, we examine the third prediction—namely, that the central location of the SGS is critical for efficient replication. The SGS was displaced from its central location using two different approaches, and the effects on Mu replication were monitored. In one, the SGS was reinserted at various locations in a prophage lacking the central SGS. In a second approach, deletions were used to alter the relative location of the SGS in relation to the Mu ends.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. Lysogens were constructed in *E. coli* K-12 AB1157 *recB recC sbcB* (Table 1) using Mu *cts62*. Plasmid pMP1523 has a fragment of Mu DNA [*Bam*HI (17.2 kb)–*Cla* I (18.6 kb)] cloned in the *Sca* I site of pBR322. Plasmids containing cloned fragments of Mu DNA in the pKN series were generously supplied by W. Schumann (4).

Isolation of Short and Long SGS-Containing Fragments. The *Bam*HI (17.2 kb)–*Sca* I (18.15 kb) fragment of Mu DNA was cloned into the *Eco*RI site of a pBR322 plasmid that had the *Dra* I sites at 3232 and 3251 replaced by a *Xho* I site (kindly supplied by D. Berg). The Mu fragment and the *Eco*RI-cleaved plasmid were end-filled with Klenow polymerase and joined by blunt-end ligation. In the selected clone, the *Sca* I end of the fragment was adjacent to the ampicillin-resistance gene (ApR) and the *Bam*HI end was adjacent to the tetracycline-resistance gene. Cleavage with *Xho* I and *Mlu* I generated the short fragment of ≈1.3 kb consisting of the 1.1-kb ApR linked to 147 bp of Mu containing the SGS. Cleavage with *Xho* I and *Hin*dIII generated the long fragment of ≈2.1 kb consisting of the ApR linked to 950 bp of Mu containing the SGS and an additional 29 bp of pBR322 (from *Eco*RI to *Hin*dIII).

Construction of Prophages with Translocated SGS. The short and long fragments were blunt-end ligated into desired sites in fragments of Mu DNA carried on appropriate plasmids (pKN35 for the *Hpa* I site at 21.5 kb; pKN48 for the *Hin*dIII site at 26.7 kb and the *Sal* I site at 30.4 kb; pKN50

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Abbreviations: SGS, strong gyrase binding site; ApR, ampicillin-resistance cassette; KnR, kanamycin-resistance cassette.

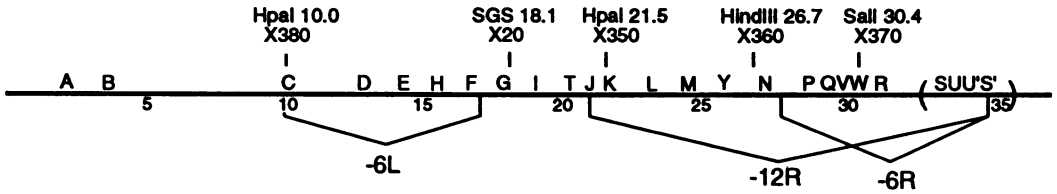

FIG. 1. Physical/genetic map of the bacteriophage Mu genome. Restriction enzyme sites used for moving the SGS and approximate locations of deletions used in the experiments below are indicated.

for the *Hpa* I site at 10.0 kb; pMP1523 for the *Sma* I site in the Kn cassette replacing the SGS deletion at 18.0–18.15 kb). The resulting plasmid DNAs were linearized and transformed into the lysogen X20 or X280. Selection for ApR yielded the recombinant prophages in the strains listed in Table 1. The structures of all prophages were verified by restriction digestion and Southern analysis.

Construction of Deleted Prophages. Plasmids carrying cloned fragments of Mu DNA were cleaved with restriction enzymes to delete regions of the Mu DNA, and either a kanamycin or ampicillin cassette was inserted at the site of each deletion. The resulting plasmid DNA was transformed into the appropriate lysogen and recombinant prophages were selected, as described above. Plasmid pKN82 was used for the right-end 5.9-kb deletion (X400), pKN27 for the left-end 6.1-kb deletion (X402), a combination of pKN50 and pKN48 for the right-end 11.8-kb deletion (X401) and for the 18.9-kb central deletion (X405), and a combination of pKN50 and pKN82 for the central 19.1-kb deletion (X406).

Measurement of Mu DNA Replication. Cultures of lysogens were grown in L broth at 30°C to $\approx 10^8$ cells per ml and induced by shifting to 42°C. Samples of 2 ml were labeled at intervals for 3 min with 0.1 mCi of [^{3}H]thymidine (1 Ci = 37 GBq). Labeled DNA was isolated and hybridized to filters with bound Mu DNA as described (5) or to filters with a 5.1-kb left-end *Eco*RI fragment of Mu DNA that is common to all of the prophages used. The latter filters were used when replication of prophages of different lengths were compared; use of filters with full-length Mu DNA would underestimate replication of deleted prophages. The data are expressed as the percent of input counts per ml bound to the filters (5).

RESULTS

Restoration of the Deleted SGS. A prophage lacking the SGS was originally constructed by deleting 147 bp from the center of the prophage (18.0–18.15 kb) and inserting a 1.3-kb KnR (3). We wished to determine if a DNA fragment containing the SGS could be reinserted into the center of the deleted prophage and restore replication and then to determine the effect on replication of inserting the SGS-containing fragment at other sites in the deleted prophage.

Table 1. Bacterial strains

X0	AB1157 *recB recC sbcB*
X1	X0/Mu *cts62*
X20	X0 mal::Mu *cts62*
X280	X20 Δ[*Mlu* I (18.0 kb)–*Sca* I (18.15 kb)] + KnR
X340	X280 + short fragment at *Sma* I site of KnR
X341	X280 + long fragment at *Sma* I site of KnR
X344	X280 + short fragment at *Sma* I site of KnR (− orientation)
X350	X280 + short fragment at *Hpa* I site (21.5 kb) of Mu
X360	X280 + short fragment at *Hin*dIII site (26.7 kb) of Mu
X370	X280 + short fragment at *Sma* I site (30.4 kb) of Mu
X380	X280 + short fragment at *Hpa* I site (10.0 kb) of Mu
X400	X1 Δ[*Hpa* I (27.4 kb)–*Hpa* I (34.6 kb)] + KnR
X401	X1 Δ[*Hpa* I (21.5 kb)–*Hpa* I (34.6 kb)] + KnR
X402	X1 Δ[*Hpa* I (10.0 kb)–*Bam*HI (17.2 kb)] + ApR
X403	X400 Δ[*Hpa* I (10.0 kb)–*Bam*HI (17.2 kb)] + ApR
X404	X401 Δ[*Hpa* I (10.0 kb)–*Bam*HI (17.2 kb)] + ApR
X405	X1 Δ[*Bam*HI (14.5 kb)–*Hpa* I (34.6 kb)] + KnR
X406	X1 Δ[*Hpa* I (10.0 kb)–*Sma* I (30.4 kb)] + KnR

ApR, ampicillin-resistance cassette; KnR, kanamycin-resistance cassette.

In our initial experiments, the 147-bp *Mlu* I–*Sca* I fragment was used to supply the SGS (short fragment). Within the 147-bp fragment, the gyrase cleavage site is 43 bp from the *Mlu* I cleavage site (3), raising the concern that the short fragment might lack some portion of the SGS. Hence, in addition, a larger *Bam*HI–*Sca* I fragment of about 850 bp (17.2–18.15 kb) was used to supply the SGS (long fragment).

To determine if the fragments can restore replication of the deleted prophage, the short and long fragments were each recombined together with a selectable ApR back into the center of the genome of the deleted prophage in strain X280 as described in *Materials and Methods*. Each fragment was inserted in the same orientation as the original deleted sequence and, in addition, the short fragment was inserted in the opposite orientation.

Lysogens containing the newly constructed prophages with the fragments reinserted at the center of the genome were induced, and the kinetics of lysis and DNA replication were determined for each lysogen (Fig. 2). Lysis with the long and short fragments showed relatively short delays of about 5 min and 10 min, respectively, compared to the parental X20, independent of the orientation of the SGS. Replication patterns mirrored the lysis patterns, showing near-normal restoration of replication with insertion of the long fragment and slightly lower levels with insertion of the short fragment. As a control, the KnR or ApR was individually inserted into several locations in the wild-type prophage, including the *Mlu* I site (18.0 kb) and *Sca* I site (18.15 kb), and in both orientations at some sites, and the effects on lysis and replication were monitored. Delays of 5 min or less in these parameters were observed for all of the constructs (ref. 3; data not shown).

We conclude that both the short and long fragments can supply a functional SGS and restore replication and that the longer fragment may carry a slightly more efficient site. The observed delays in lysis and replication are minimal, considering that insertion of the cassettes by themselves resulted in delays of about 5 min, and particularly when compared with the effects of other conditions reported below.

Movement of the SGS to Other Locations. To determine the effect of inserting the SGS at other locations in the Mu prophage, the short fragment was recombined together with an ApR marker at various locations between the center and right end of the prophage in X280: *Hpa* I, 21.5 kb; *Hin*dIII, 26.7 kb; *Sal* I, 30.4 kb (Fig. 1). The resulting cultures were induced and lysis and replication were monitored (Fig. 3). Lysis and replication in each of the lysogens with a displaced SGS were markedly delayed, and the extent of the delay was dependent on the location of the SGS. The length of the delay increased with increasing distance between the location of the SGS and the center of the genome. For example, rapid lysis of the parental X20 (SGS at 18.1 kb) began about 40 min after induction, while lysis of X360 (SGS at 26.7 kb) began

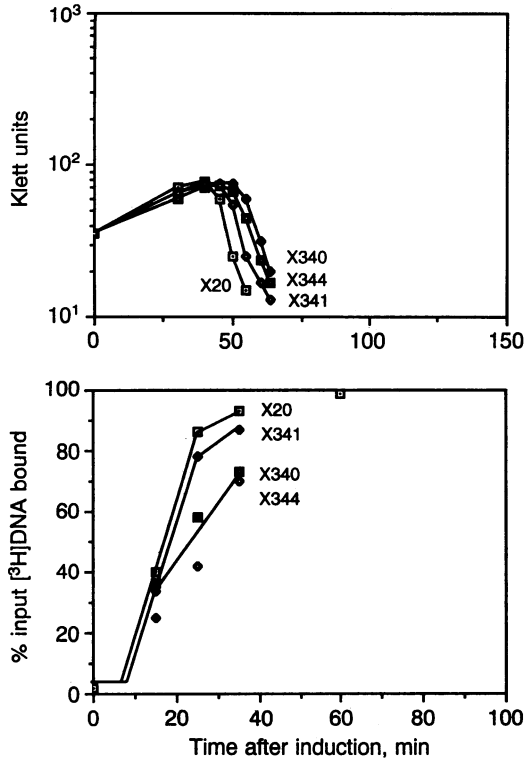

FIG. 2. Insertion of the SGS at the prophage center. Cultures of lysogens were grown in L broth at 30°C to about 1×10^8 cells per ml and then induced by shifting to 42°C. Growth was monitored with Klett readings and samples were pulse-labeled at intervals with [3H]thymidine for 3 min to measure DNA replication. The prophage in X20 is wild type; the prophages in X340, X341, and X344 contain a deletion of the SGS and insertions of the short fragment, long fragment, and short fragment (inverted orientation), respectively. (*Upper*) Growth and lysis of cultures. (*Lower*) Mu DNA replication.

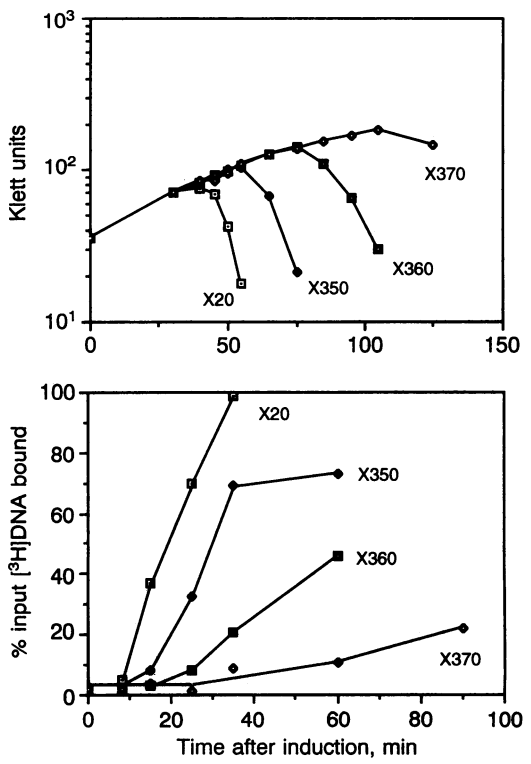

FIG. 3. Insertion of the SGS (short fragment) in the prophage right half. Lysogens were induced as in Fig. 2. The prophages in X350, X360, and X370 contain a deletion of the SGS and insertions of the short fragment at *Hpa* I (21.5 kb), *Hin*dIII (26.7 kb), and *Sma* I (30.4 kb), respectively. (*Upper*) Growth and lysis of cultures. (*Lower*) Mu DNA replication.

about 80 min after induction and very slow lysis of X370 (SGS at 30.4 kb) began about 120 min after induction. Parallel delays in replication were observed in these lysogens.

A second set of strains was constructed by inserting the long fragment at the same sites as those used in Fig. 3. The kinetics of lysis and replication were again measured after induction, and the results were almost identical with those obtained using the short fragment (data not shown).

To examine whether similar results would be obtained with insertion of the SGS on the left side of the prophage lacking the SGS, the short fragment was inserted at the *Hpa* I site at 10.0 kb. In this culture, lysis is not informative because the insertion interferes with the synthesis of the C protein, which is required for activation of late transcription and, hence lysis. However, replication was greatly reduced from the control, consistent with the results from insertions on the right side of the genome (Fig. 4).

Displacement of the SGS by Deletion. An alternative approach to distort the symmetrical placement of the SGS between the termini without actually moving the SGS from its immediate context is to delete DNA from one half of the prophage. The model described above predicts that deletions lead to asymmetry and should inhibit replication, with larger deletions leading to greater asymmetry and more severe effects on replication. Two deletions of approximately 6 kb and 12 kb from the right half of the prophage in X20 (Fig. 1) were constructed (the sizes of the deletions are the net results of deleting Mu DNA and inserting an antibiotic-resistance cassette), and the effects on prophage replication were determined. Our routine assay for Mu replication, which involves annealing labeled DNA from pulse-labeled cultures to nitrocellulose filters containing Mu DNA, was modified to account for the different lengths of Mu DNA in the deleted prophages; a short fragment of Mu DNA, common to all the deleted prophages, was used on the filters (see *Materials and Methods*). Replication following induction of X400 carrying the prophage with a 6-kb deletion (Fig. 5 *Upper*) was reduced relative to that in the parental X20, and replication in X401 carrying the prophage with a 12-kb deletion (Fig. 5 *Upper*) was severely reduced.

If the reduction in replication observed with the deleted prophages is due to displacement of the SGS from the center of the prophage, then an additional deletion in the left half of the prophage, which places the SGS closer to the center of the doubly deleted prophage, might increase replication. Accept-

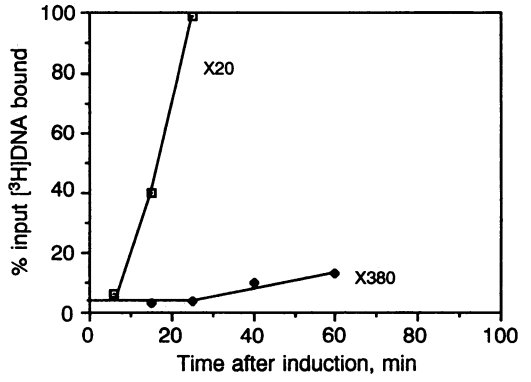

FIG. 4. Insertion of the SGS (short fragment) in the prophage left half. Lysogens were induced as in Fig 2. The prophage in X380 contains a deletion of the SGS and insertion of the short fragment at *Hpa* I (10.0 kb). Only DNA replication is presented, as the insertion abolishes lysis.

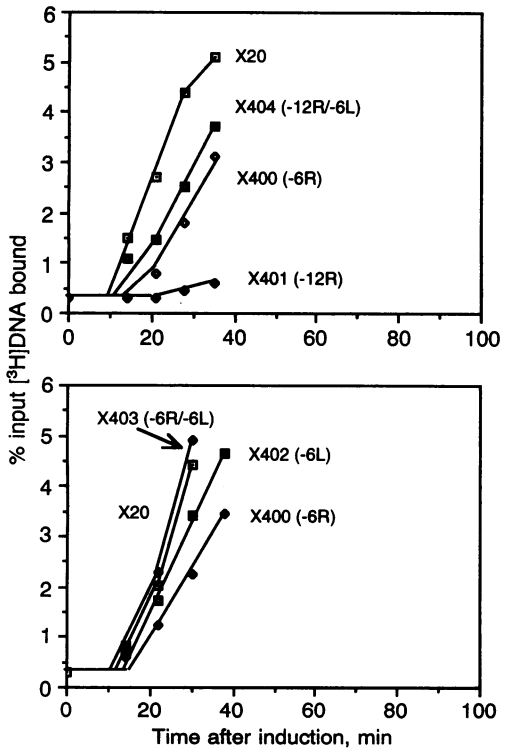

FIG. 5. Deleted prophages that retain the SGS. Lysogens were induced as in Fig. 2 and Mu DNA replication was measured. (*Upper*) The prophages in X400 and X401 contain deletions of about 6 kb and 12 kb from the right half, respectively; the prophage in X404 contains both a 12-kb deletion from the right half and a 6-kb deletion from the left half. (*Lower*) The prophages in X400 and X402 contain deletions of about 6 kb from the right half and the left half, respectively; the prophage in X403 contains both a deletion of 6 kb from the right half and a deletion of 6 kb from the left half.

able deletion in the left half of the molecule is limited to regions beyond about 10 kb, since functions encoded in the leftmost 10 kb can influence the rate of replication (6). Deletion of ≈6 kb from the left half of the prophage in X402 resulted in a decrease in replication (Fig. 5 *Lower*). When the left-half 6-kb deletion was paired with the right-half 6-kb deletion (X403; Fig. 5 *Lower*), the resulting replication rate was restored to approximately wild-type level; when paired with the right-half 12-kb deletion (X404; Fig. 5 *Upper*), replication increased to approximately the level observed with a single 6-kb deletion.

Replication of Mini-Mu. Mu prophage deleted of most of the internal portions of the genome, but retaining the termini and replication functions (mini-Mu) are capable of fairly efficient replication (7). The double deletion in the prophage in X404 (12-kb right half and 6-kb left half) resulted in a large increase in replication compared to a prophage having only the right-half deletion. Could this be due to reduction of the prophage size to about 19 kb, and hence creation of an efficiently replicating mini-Mu? To answer this question, the two different prophages in X405 and X406, comparable in size to the prophage in X404, were constructed. These differ from the prophage in X404 in that they contain single deletions and remove the SGS. The replication rates of these mini-Mus, although higher than the replication rate of a full-size Mu lacking a SGS, were much lower than that of the doubly deleted prophage retaining the SGS (Fig. 6).

DISCUSSION

Mu SGS. Replication of bacteriophage Mu DNA requires that the DNA be negatively supercoiled and hence requires

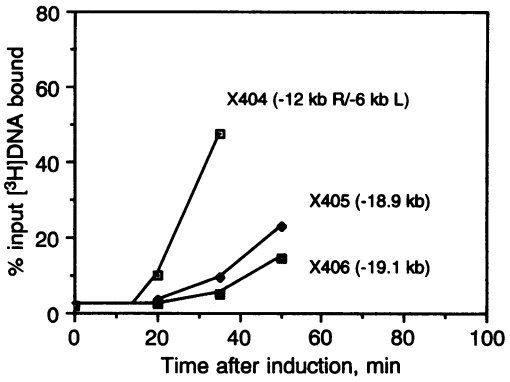

FIG. 6. Deleted prophages that do not retain the SGS. Lysogens were induced as in Fig. 2 and Mu DNA replication was measured. The prophages in X405 [Δ*Bam*HI (14.5 kb)–*Hpa* I (34.6 kb)] and X406 [Δ*Hpa* I (10.0 kb)–*Sma* I (30.4 kb)] contain large deletions that remove the SGS. The prophage in X404 is as in Fig. 5.

host DNA gyrase (1). However, our evidence indicates that gyrase has a role in addition to that of maintaining an appropriate degree of superhelicity and that that role requires a strong, centrally located binding site. A series of studies on the role of this site on the replication of Mu have revealed the following information. (*i*) An SGS is present near the center of the Mu genome—at 18.05 kb in the 37.2-genome (3). (*ii*) The SGS is required for efficient replication, as deletion of the site from a Mu prophage or an insertion into the site results in very long lags in replication following induction of the appropriate lysogens (3). Studies with dilysogens have demonstrated that the defective prophage cannot be complemented in trans by a wild-type prophage (unpublished data). (*iii*) The *nuB* mutants of Mu, which are able to grow on an *E. coli gyrB* mutant that does not allow the growth of wild-type Mu (8), possess single-base changes in the SGS, resulting in more efficient gyrase binding and cleavage at the site (3). (*iv*) Inhibition of replication of a prophage lacking the SGS occurs at a step in the replication pathway *after* early transcription and the synthesis of required Mu replication proteins and *before* strand transfer or nicking at the Mu termini (unpublished data).

The present studies were designed to determine the effects of displacing the SGS from its central location in the Mu genome. In two sets of experiments described here displacement resulted in decreased rates of Mu replication after induction of lysogens carrying altered prophages.

In the first set of experiments, the SGS was inserted at various locations in a prophage lacking the central SGS. The further the location of the SGS was from the center of the prophage, the greater was the inhibition of replication. We interpret these results as demonstrating the essentiality of a centrally located gyrase binding site for optimal replication. Other possibilities include (*i*) gyrase bound at the SGS might have to interact with a second protein bound nearby, and displacement of the SGS would increase the difficulty of forming this interaction and (*ii*) displacement of the SGS may have placed it in different contexts in which the efficiency of gyrase binding is decreased.

Displacement of the SGS from the prophage center also was accomplished by deleting DNA between the SGS and one of the termini of the prophage. The observation that deletions reduce Mu replication and that larger deletions have greater effects is again consistent with the need for central location of the SGS. These experiments are not as open to the alternative explanations listed above, as the immediate context of the SGS remains unchanged. Moreover, the deletions are unlikely to have removed sites that enhance replication

because paired deletions flanking the SGS lead to higher rates of replication than single deletions.

The present results complement the previous insertion data (2) and are not subject to the possible criticism that the effects of inserted DNA could be due to the *nature* of the DNA rather than to the *length* of the DNA. Indeed, while examining various drug-resistance cassettes for the present studies, we found that insertion of cassettes for gentamycin resistance or for chloramphenicol resistance into the Mu prophage resulted in reduced rates of replication (unpublished data). The mechanism of these effects is not known, but they do emphasize the need to cautiously interpret the insertion data.

The results with all of the modified prophages, with or without the SGS, can be explained by the following notion—synapsis of the termini of small mini-Mu prophages may be fairly efficiently accomplished by random collision or "slithering" on supercoiled DNA. However, as the length of the prophage DNA increases, synapsis of the termini in the prophage, subject to whatever constraints exist within the compacted bacterial nucleoid, becomes increasingly inefficient. This is consistent with results of Tn*10* transposition carried out by Morisato *et al.* (9), who showed that increasing the length of DNA between Tn*10* ends decreased transposition efficiency by 35% per kb, and with the results on length dependency of IS*1* transposition (10). A wild-type prophage of 37 kb may, therefore, require an active mechanism for synapsis, and that mechanism would require the central location of the SGS. We suggest that the role of the SGS is to orient the supercoiling of prophage DNA to place the SGS at the apex of an extended supercoiled loop and to bring the prophage termini into proximity. Once the termini are in proximity to each other, they can be synapsed by transposase molecules bound at each terminus (11). One of the intriguing questions raised by these considerations is whether the prophage termini are synapsed in the lysogen by repressor proteins bound at the termini, as repressor and transposase share binding properties for the termini (11).

A similar mechanism has been shown to function in the organization of small interwound plasmid DNA *in vitro* by the localization of bent DNA sequences to the apex of an interwound loop (12). This result raises the question of whether active gyration at the SGS is required for function or whether the extreme bend induced by the binding of gyrase (13) is sufficient; our preliminary evidence suggests that merely bending prophage DNA lacking the SGS at its center does not restore replication (unpublished data).

Chromosomal Supercoiled Domains. Once the Mu ends are synapsed, the supercoiled prophage DNA may resemble one of the independently supercoiled domains of the *E. coli* chromosome. Our current understanding of the *in vivo* structure of bacterial chromosomes is fragmentary, but evidence from several different experimental approaches suggests that the nucleoid contains about 50–100 independently supercoiled domains (14–16). Many questions remain about the complex structure of the *E. coli* nucleoid. Are supercoiling domain boundaries fixed or plastic? What are the elements involved in maintaining a domain? A role for gyrase in organizing nucleoid structure has been discussed by several investigators (17–19) and our results suggest to us that gyrase may be able to function as an organizing agent for the folded chromosome, perhaps forming the apex of loops seen in electron micrographs of gently lysed cells.

I wish to thank my colleague Pat Higgins for his continued involvement in this project and Mark Karlok for outstanding technical assistance. This work was supported by National Science Foundation Grant DMB-9018328 to M.L.P.

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