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Characterization of Mu Prophage Lacking the Central Strong Gyrase Binding Site: Localization of the Block in Replication

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Bacteriophage Mu contains an unusually strong DNA gyrase binding site (SGS), located near the center of its genome, that is required for efficient Mu DNA replication (M. L. Pato, Proc. Natl. Acad. Sci. USA 91:7056–7060, 1994; M. L. Pato, M. M. Howe, and N. P. Higgins, Proc. Natl. Acad. Sci. USA 87:8716–8720, 1990). Replication of wild-type Mu initiates about 10 min after induction of a lysogen, while replication in the absence of the SGS is delayed about an hour. To determine which step in the replication pathway is blocked in the absence of the SGS, we inactivated the SGS by deletion and by insertion and studied the effects of these alterations on various stages of Mu DNA replication. Following induction in the absence of a functional SGS, early transcription and synthesis of the Mu-encoded replication proteins occurred normally. However, neither strand transfer nor cleavage at the Mu genome termini could be detected 40 min after induction. The data are most consistent with a requirement for the SGS in the efficient synapsis of the Mu prophage termini to form a separate chromosomal domain.

Amplification of bacteriophage Mu DNA following infection of a sensitive host or induction of a Mu lysogen is accomplished by a series of replicative transposition events (for reviews, see references 15 and 21). Host-encoded requirements for this process include the DNA replication proteins and proteins which affect DNA structure: IHF, HU, and gyrase. The phage-encoded requirements include the A (transposase) and B proteins, the termini of the Mu genome, which carry binding sites for the transposase (3), and an enhancer sequence located near the left terminus which is involved in correct alignment of the termini (10, 19, 29). In addition, an unusually strong DNA gyrase binding site (SGS), located near the center of the Mu genome, is required for efficient Mu replication (22, 23). Deletion of 147 bp containing the gyrase binding site from a Mu prophage results in a delay of replication for about an hour after induction.

The host DNA gyrase, along with topoisomerase I, is required for maintaining a proper degree of superhelicity in cellular DNA (for a review, see reference 4), and alterations in superhelicity have been implicated in the regulation of gene expression in a large number of biological systems (for a review, see reference 5). However, the existence of unusual gyrase binding sites, such as the Mu SGS and a site in pSC101 that is required for plasmid maintenance (32), indicates that gyrase may play a more specific role in some biological processes. Mu requires gyrase for replication (24, 37), but what specific requirements gyrase fulfills and what role the SGS plays compared with numerous weaker sites in the Mu genome remain to be elucidated.

There are several stages at which replication of prophage lacking the SGS could be inhibited. These include (i) early transcription and translation, which yield the required A and B proteins; (ii) synapsis of the prophage termini through oligomerization of transposase molecules bound at the termini (18); (iii) formation of a "cleaved-donor complex" (3) (also called type I transpososome [28]) by introduction of single-strand cleavages at the Mu termini; (iv) formation of a "strand transfer complex" (16) (also called type II transpososome [28]), which includes synapsis with target DNA and strand transfer; and (v) binding of host replication machinery and replication itself (7). In this study we characterized Mu prophages with a deleted or altered SGS, and here we describe the effects of the changes on various stages of the replication pathway.

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MATERIALS AND METHODS

Bacteria, bacteriophage, and plasmids. Strain X1 is *Escherichia coli* K-12 AB1157 that is *recB recC sbcB* and monolysogenic for Mu*cts*62. MP1594 is W3110 *thy lacZ*::Mu*cts*62. MP507 is W3110 *thy malB*::Mu *B*Am1066. The phages used were Mu*cts*62, Mu pAp1, and Mu *nu*B1 (37). Plasmid pBR322 was used for cloning of Mu DNA fragments. Cultures were grown in L broth, except for the experiments requiring thymine starvation, when T medium supplemented with 20 μg of thymine per ml and 0.4% glucose was used (13).

Prophage constructions. Construction of the prophage with a deletion of 147 bp containing the SGS (*Mlu*I-*Sca*I; 18.0 to 18.15 kb) and an insertion of a kanamycin resistance (Kn^r) cassette to create X180 was described previously (23). To construct a prophage with an insertion in the SGS, a 1.4-kb *Bam*HI-*Cla*I DNA fragment from Mu *nu*B1 (17.2 to 18.6 kb) with ligated *Bam*HI linkers was cloned into pBR322 that had been cleaved with *Eco*RI and *Bam*HI and had *Bam*HI linkers added. The resulting plasmid was cleaved with gyrase and enoxacin as described previously (23), the ends were filled in with DNA polymerase, and an 8-bp *Xho*I linker was inserted. After cleavage with *Xho*I and end filling, a 1.3-kb Kn^r cassette was inserted. The plasmid DNA was cleaved with *Bam*HI and transformed into strain X1 to recombine the insertion into the resident prophage. Kanamycin-resistant recombinants were isolated, and their structures were verified by Southern analysis. The lysogen containing the Mu prophage with an *Xho*I linker and a Kn^r cassette at the SGS was designated X190.

Insertion of the Kn^r cassette at the *Sca*I site (18.15 kb) adjacent to the SGS was performed in a similar manner. A pBR322 plasmid containing the *Bam*HI-*Cla*I fragment of Mu DNA (17.2 to 18.6 kb) cloned into the *Sca*I site of the plasmid was cleaved with *Sca*I, and the Kn^r cassette was inserted. The resultant plasmid was linearized with *Eco*RI and transformed into X1. The appropriate recombinant lysogen containing a Mu prophage with a Kn^r cassette at the *Sca*I site was designated X200.

The structures of the recombinant prophages were verified by Southern analysis. Genomic DNA was prepared, cleaved with *Eco*RI and *Bam*HI, electrophoresed on 0.7% agarose gels, and transferred to membrane filters. The filters were probed with ^{32}P-labeled Mu DNA and subjected to autoradiography. The presence of DNA fragments of the predicted sizes was verified.

Measurement of DNA replication and transcription. DNA and RNA syntheses were measured by pulse-labeling 2-ml samples of the induced cultures for 2 min with 50 mCi of either [^{3}H]thymidine or [^{3}H]uridine per ml, respectively, and

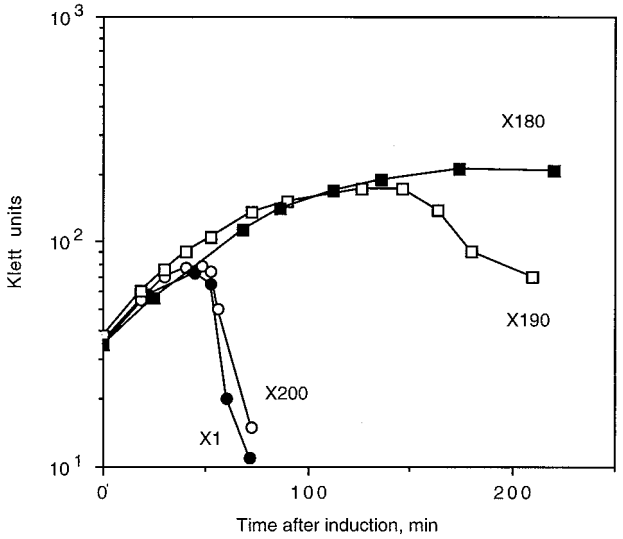

FIG. 1. Lysis of induced lysogens. Cultures were grown in L broth at 30°C to a density of about 10^8 cells per ml and then induced by transfer to 42°C. Growth of the cultures was monitored by Klett readings.

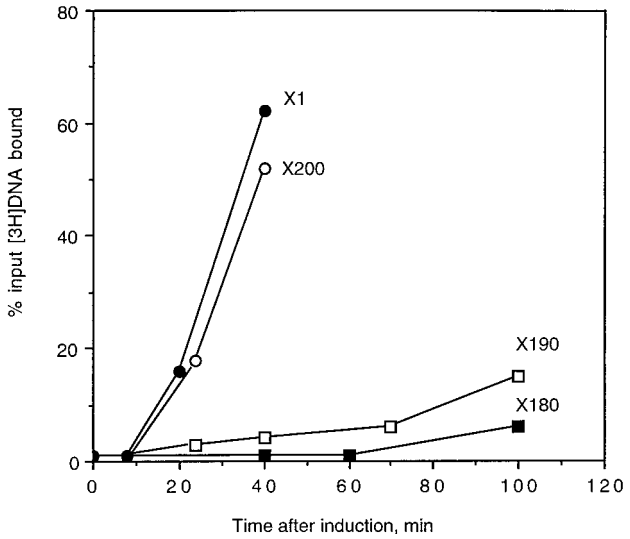

FIG. 2. Mu DNA replication. Cultures were induced as for Fig. 1, and 2-ml samples were pulsed-labeled at intervals for 2 min with 50 mCi of [^{3}H]thymidine per ml. DNA was isolated and annealed to filters containing immobilized Mu DNA. The results are expressed as percentages of input counts per minute bound to the filters in the annealing assay.

hybridizing labeled DNA or RNA to filters containing immobilized Mu DNA as described previously (20, 31). The results are expressed as the percentages of input counts per min in the assay that are bound to the filters.

PFGE. Preparation of DNA samples for pulsed-field gradient electrophoresis (PFGE) was performed essentially as described previously (25). Cells harvested before and after thermoinduction were lysed in agarose blocks, and the DNA was cleaved with *Not*I. DNA fragments were separated by transverse alternating-field electrophoresis in a 0.85% agarose gel by using 20-s pulses at 150 mA for 16 to 18 h.

Electrophoresis of single-strand DNA. DNA was denatured in 1 M glyoxal–50% dimethyl sulfoxide at 50°C for 1 h and separated on a 1% agarose gel in 10 mM sodium phosphate, pH 7.0, at 40 mA for 5 h, with recirculation of the running buffer. Denaturation was monitored by staining with ethidium bromide, which yields a red fluorescence with single-strand DNA at 640 nM.

RESULTS

Induction of lysogens. The Mu*cts*62 prophage of strain X1 was modified by substitution of 147 bp containing the SGS with a Kn^r cassette to produce strain X180, by insertion of an *Xho*I linker and a Kn^r cassette into the SGS to produce strain X190, and by insertion of a Kn^r cassette adjacent to the SGS to produce strain X200 (see Materials and Methods). The lysogens were thermoinduced by growing cultures to a density of 10^8 cells per ml at 30°C in L broth and then shifting the culture to 42°C. The parental lysogen, X1, lysed approximately 50 min after induction (Fig. 1), with the production of about 5×10^9 plaque-forming particles per ml. X180, with a deletion of the SGS, generally showed little or no lysis for several hours after induction, and no plaque-forming particles ($<10^3$/ml) were detected. X190, with an insertion in the SGS, showed partial lysis after 2 h at 42°C. Up to 5×10^8 small or pinpoint plaques per ml, depending on the indicator strain used, were observed at 4 h after induction. Lysis of X200, with a Kn^r cassette inserted adjacent to the SGS, was delayed by about 5 min, and phage titers were comparable to those of the wild type.

To measure Mu DNA replication after induction, samples of the induced cultures were pulse-labeled with [^{3}H]thymidine for 2 min at intervals. DNA was isolated from the samples and annealed to nitrocellulose filters containing bound Mu DNA, and the ratio of labeled Mu DNA annealing to the filters to total input labeled DNA was determined. Mu*cts*62 DNA replication in the parental lysogen, X1, increased rapidly about 10 min after induction, and by 30 min most of the replication in the cell was of Mu DNA (Fig. 2). Replication of X180 was virtually undetectable during the first hour after induction, although by 100 min significant replication was observed. Replication of X190 was greatly reduced compared with that of X1 but was slightly higher than that observed with X180. Insertion of the Kn^r cassette in X200 resulted in only a slight decrease in the replication rate. Hence, inactivation of the SGS by deletion or insertion severely inhibits replication.

Although no plaque-forming particles were obtained from induced cultures of X180, when a culture induced for 5 h was treated with chloroform and then centrifuged at 20,000 $\times$ *g* for 3 h, particles which contained DNA with a restriction pattern expected for the X180 prophage were isolated (data not shown). The amount of DNA obtained was roughly comparable to that obtained with a wild-type Mu lysogen which lyses in about 50 min. The X190 and X200 lysates could be titered and were used to determine the effect of the insertion into the SGS on the frequency of lysogenization. A sensitive host was infected with each and plated on L broth plus 50 mg of kanamycin per ml after an hour of incubation to express drug resistance, and the number of kanamycin-resistant lysogens was determined. The frequency of lysogenization was approximately 1% for both phage. We therefore conclude that the SGS is not required for efficient lysogenization of the infecting Mu phage.

Early transcription and translation. The inhibition of Mu replication resulting from deletion of, or insertion into, the SGS could result from interference with any of the several steps leading up to replication. The initial required events after induction are early transcription and synthesis of the Mu A and B proteins. To determine if transcription supplying these functions is affected in X180, transcription was measured by pulse-labeling with [^{3}H]uridine at intervals after induction and hybridizing the labeled RNA to Mu DNA bound to filters. The data in Fig. 3 show that early transcription in X180 was similar to that in the parent, X1. Late transcription, which requires prior Mu DNA replication (34), was severely reduced relative to that of the control, as expected.

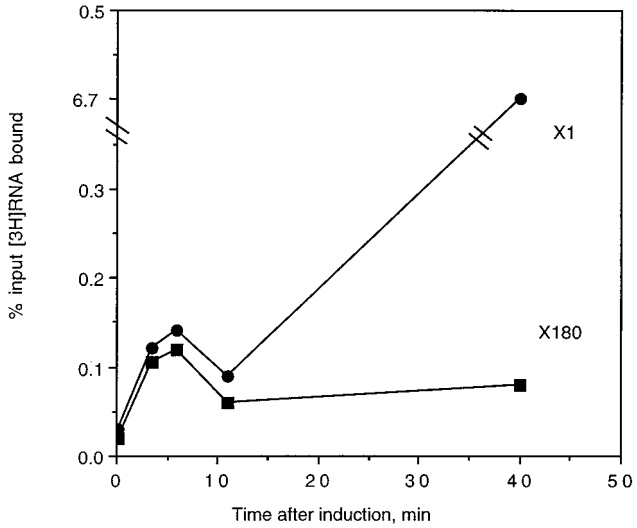

FIG. 3. Mu transcription. Cultures were induced as for Fig. 1, and 2-ml samples were pulse-labeled at intervals for 2 min with 50 mCi of [^{3}H]uridine per ml. RNA was isolated and hybridized to filters containing immobilized Mu DNA. The results are expressed as percentages of input counts per minute bound to the filters in the hybridization assay.

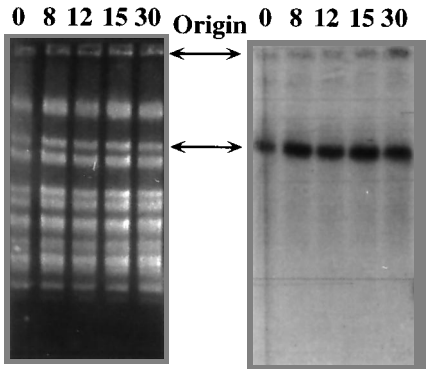

FIG. 4. PFGE analysis of Mu *B*Am1066. A culture of a *B*Am1066 lysogen, MP507, was induced and sampled at intervals (indicated at top in minutes). The cells were embedded and lysed in agarose blocks, and DNA was cleaved with *Not*I. DNA fragments were separated by transverse alternating-field electrophoresis in a 0.85% agarose gel by using 20-s pulses at 150 mA for 16 to 18 h. After electrophoresis, the gel was stained with ethidium bromide and photographed (left panel). DNA fragments were transferred to a nitrocellulose filter, annealed with ^{32}P-labeled Mu DNA, and autoradiographed (right panel). The top arrow indicates the position of the gel origin, and the bottom arrow indicates the position of the Mu DNA-containing fragment.

To ensure that translation of the early transcript was occurring, two experiments were performed. First, killing of the host was monitored and was shown to occur rapidly following induction of X180 (data not shown). In the absence of DNA replication, killing is due to the expression of the *kil* gene encoded immediately downstream of the B gene (30); hence, the early transcript is translated. Second, the ability of Mu X180 to complement a Mu B amber prophage was examined. An F′ plasmid carrying Mu*cts*62 *B*Am1066 was introduced into X180; induction resulted in lysis within an hour, with production of 10^9 amber phage particles per ml. Hence, Mu X180 was able to supply sufficient B protein to complement growth of the B amber prophage.

To further examine whether a replicating Mu prophage can complement a second prophage lacking a functional SGS, a dilysogen was constructed with Mu pAp1 (SGS^+) and Mu X180. A culture of the dilysogen was induced, and lysis was observed by about 50 min. To determine the identity of the phage particles in the lysate, DNA was isolated from the particles and analyzed by restriction analysis. The gels showed the restriction fragments predicted for Mu pAp1, with no indication of the fragments expected from Mu X180 (data not shown). Hence, most or all of the particles contained Mu pAp1 DNA, and we can conclude that replication of Mu X180 was not successfully complemented by Mu pAp1.

One might expect that the unreplicated Mu X180 prophage in the induced dilysogen would be packaged along with the approximately 100 replicated copies of Mu pAp1 per cell. If so, approximately 1% of the lysate would be Mu X180. Although this level of Mu X180 would not be detected by the above-described restriction analysis, if could be determined genetically. Since the loss of the SGS apparently does not affect lysogenization (see above), the lysate was used to infect a sensitive strain of *E. coli* at a multiplicity of infection of 0.01 to look for new lysogens. After an hour of incubation at room temperature to allow expression of drug resistance, the cells were plated on Luria-Bertani agar containing 50 μg of ampicillin per ml, to select for Mu pAp1 lysogens, or 25 μg of kanamycin per ml, to select for Mu X180 lysogens. Kanamycin-resistant lysogens were observed at a frequency of 6×10^3 cells per ml, whereas ampicillin-resistant lysogens were found at a frequency of 6×10^5 cells per ml. When 30 independent ampicillin- or kanamycin-resistant cells were picked and tested on both types of media, they were found to be resistant only to the drug for which they were initially selected. Thus, most of the lysogens bear only one type of prophage. We conclude that phages carrying the 147-bp deletion can be packaged if Mu late genes are expressed, and they can efficiently infect a new host and make lysogens. Their abundance in the lysate is approximately what would be expected if unreplicated X180 prophage were passively packaged along with the newly replicated Mu pAp1 copies.

The strand transfer reaction. The strand transfer reaction resulting in ligation of Mu termini to target DNA is readily assayed in vitro (16, 28). For analysis of in vivo strand transfer we developed a technique using PFGE. The technique is based upon the observation that large chromosomal fragments containing branched structures formed by Mu strand transfer fail to enter a pulsed-field gel (14, 23a).

The results of induction under conditions expected to inhibit Mu DNA replication (i) before strand transfer—in this case, use of a B^- prophage—and (ii) after strand transfer—induction during thymine starvation—are presented in Fig. 4 and 5, respectively. Appropriate lysogens were analyzed by PFGE before induction and at intervals after induction. Cells were embedded and lysed in agarose, and total DNA was cleaved with the restriction enzyme *Not*I, which does not cleave within Mu DNA. The resulting fragments were separated by PFGE, and Southern analysis of the gel after electrophoresis identified a fragment of about 400 kb as the Mu DNA-containing *Not*I fragment in the uninduced cells. With the B^- mutant, the 400-kb fragment observed before induction in both the ethidium bromide-stained gel and in the Southern blot persisted, indicating that efficient strand transfer had not occurred in the intervals examined (Fig. 4). When strand transfer did occur, following induction in the absence of required thymine, the 400-kb band was not present after induction; instead, the Mu DNA-containing fragment was retained at the gel origin (Fig. 5). Inhibition of replication after strand transfer also was observed by the PFGE technique with a *clp*X lysogen (14).

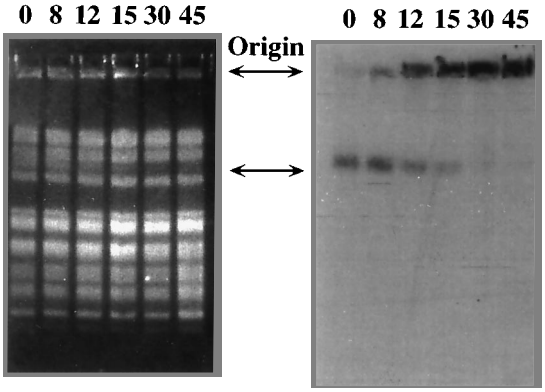

FIG. 5. PFGE analysis of thymine starvation. A culture of a thymine-requiring Mu*cts*62 lysogen, MP1594, growing in T medium plus thymine was filtered, resuspended without thymine, and induced. Samples were taken at intervals and processed as for Fig. 4. The arrows are the same as in Fig. 4.

To examine the effect of deletion of the SGS on strand transfer, the X1 and X180 lysogens were analyzed by the PFGE technique. In the X1 samples, the Mu DNA-containing band observed before induction both in the ethidium bromide-stained gel and in the Southern blot was not present after induction; instead, Mu DNA, amplified by replication, appeared at the gel origin (Fig. 6). At later times, Mu DNA-containing fragments of random sizes also appeared throughout the gel. In the X180 samples, the Mu DNA-containing band entered the gel and persisted after induction, indicating that efficient strand transfer had not occurred in the intervals examined.

The cleaved-donor complex. Formation of the cleaved-donor complex involves synapsis of the Mu ends and introduction of single-strand cleavages at the ends of the Mu prophage (2, 28); hence, inhibition at this stage should reveal single-strand nicks at the Mu termini. The presence of such nicks should be detectable by cleavage of chromosomal DNA of a lysogen with an enzyme that yields "junction fragments" of the Mu termini and adjacent host DNA, followed by denaturation. For example, cleavage of X1 DNA with *Eco*RI generates a left-end junction fragment of about 8 kb, which includes 5.1 kb of the Mu left end. The junction fragment will persist even after

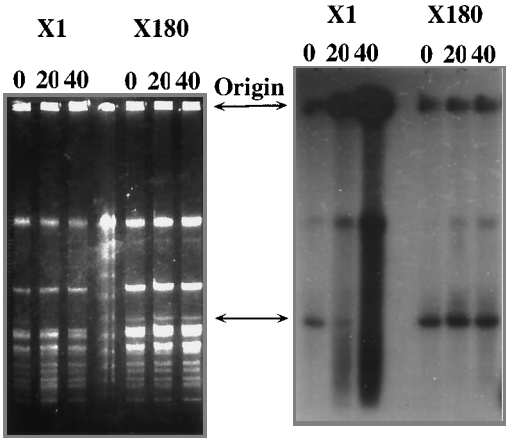

FIG. 6. PFGE analysis of prophage lacking the SGS. Cultures of X1 and X180 were sampled 0, 20, and 40 min after induction, and the samples were processed as for Fig. 4. The arrows are the same as in Fig. 4.

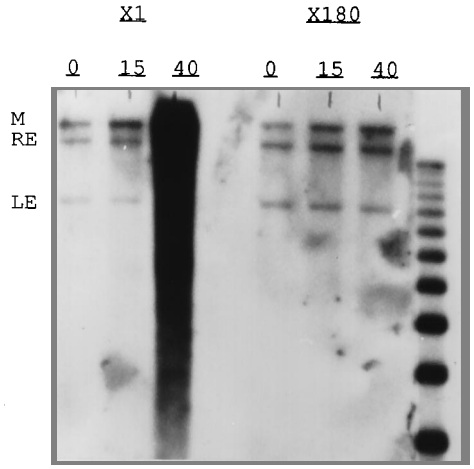

FIG. 7. Glyoxal gel electrophoresis of denatured DNA. Five-milliliter samples of cultures of X1 and X180 were taken 0, 15, and 40 min after induction. DNA was isolated and denatured in 1 M glyoxal–50% dimethyl sulfoxide at 50°C for 1 h and separated on a 1% agarose gel in 10 mM sodium phosphate, pH 7.0, at 40 mA for 5 h. DNA fragments were transferred to a nitrocellulose filter, annealed with ^{32}P-labeled Mu DNA, and autoradiographed. M, RE, and LE designate the middle fragment, right-end junction fragment, and left-end junction fragment of Mu DNA, respectively.

replication, as originally demonstrated by Ljungquist and Bukhari (11). However, if a nick has been introduced, and the subsequent strand transfer has been inhibited, denaturation should yield one single strand of 8 kb and additional strands of 5.1 and 3 kb.

DNA from X1 and X180 was isolated at intervals before and after induction, cleaved with *Eco*RI, denatured, and run on glyoxal-containing agarose gels. Southern analysis revealed 8- and ~14-kb junction fragments and an 18-kb internal Mu fragment (Fig. 7). For both strains, the 8-kb junction fragment persisted after induction without the appearance of 5- and 3-kb single-strand fragments. A range of new junction fragments is evident in the X1 samples resulting from replication. The persistence of the 8-kb fragment in induced X180 in the absence of the appearance of the cleaved fragments, along with the knowledge that strand transfer was inhibited, indicates that cleavages at the Mu termini had not been introduced.

It would be desirable to have a positive control for the above-described experiment, i.e., a condition in which single-strand cleavages occur in the absence of strand transfer. Under in vitro conditions, omission of B protein results in such an accumulation of nicked intermediates (2); hence, one might have predicted that the use of B^- lysogens would supply the desired in vivo control conditions. However, the use of B^- lysogens does not result in accumulation of nicked intermediates in vivo (1). In the absence of a positive control, the results in Fig. 7 must be interpreted cautiously. Taken together, the PFGE analysis and the glyoxal gels indicate that the inhibition of replication observed in the absence of a functional SGS occurs before formation of a strand transfer complex, or type II intermediate, and most likely before formation of a cleaved-donor complex, or type I intermediate.

DISCUSSION

Analysis of the replication pathway. We have analyzed the effect of inactivating the SGS on various stages in the pathway of Mu replicative transposition. The results show that early transcription and synthesis of the required Mu replication pro-

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Mu nuB1       5' GTGCGGG  T  }{GATT  T   TATG C   CT 3'
Mu X190       5' GTGCGGG  T  }{GATT  C  CTCG A  GA 3'
Consensus     5' RNNNRNR(T/g)}{GRYC(G/T) YNYN(G/t)NY 3'
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FIG. 8. Analysis of the sequence around the SGS. Inverted braces indicate the gyrase cleavage site. Underlined bases are those that match the proposed consensus. Bases from X190 in boldface are from the insertion of the *Xho*I linker and the kanamycin resistance cassette.

teins are unaffected by deletion of the SGS. Using techniques designed to study in vivo replication of full-length Mu DNA, we have shown that prophage with a deletion of the SGS show neither nicking at the Mu termini nor strand transfer at late times after induction. We conclude that the block in Mu replication occurs before formation of a cleaved-donor complex, or type I intermediate.

The pathway between the stage of synthesis of Mu replication proteins and the stage of formation of a cleaved-donor complex can be further subdivided. Mu A protein binds to three sites at each terminus (3) and to the IAS enhancer (10, 19, 29), and the termini are synapsed, presumably through interaction of A monomers. Transition to a stable synaptic complex (18) follows; the complex contains a tetramer of A protein monomers bound at three of the six available terminus binding sites (8, 9, 17). When Ca^{2+} is used in an in vitro reaction instead of Mg^{2+}, this stable intermediate can be isolated, without the cleavages observed in the cleaved-donor complex (18). In the presence of Mg^{2+}, single-strand cleavages at the prophage termini produce the cleaved-donor complex, or type I transpososome.

Both deletion of the SGS and insertion into the SGS inhibit replication. The degree of inhibition observed with the insertion is somewhat less than that seen with the deletion, suggesting that the binding site has been weakened rather than eliminated. Analysis of the sequence around the SGS suggests that this is reasonable, as the insertion maintains the sequence immediately around the TG cleavage site while shifting the sequence somewhat further from the consensus sequence proposed by Lockshon and Morris (12) (Fig. 8).

Role of the SGS. The data presented here and our previous demonstration that the central location of the SGS in the prophage is essential for efficient Mu replication argue in favor of a role for the SGS in synapsis of the prophage termini. To explain the earlier observation, we proposed that the role of the SGS is to organize the supercoiled structure of the prophage, placing the SGS at the apex of a separate chromosomal loop and bringing the prophage termini into proximity. The interaction of transposase molecules bound at the prophage termini and the IAS would then complete the formation of an independent prophage domain. It is conceivable that the SGS is also important in the transition to the stable synaptic complex, but evidence from studies with mini-Mu plasmids implicates the superhelicity of the DNA outside of the prophage termini, rather than within the prophage, in this transition (33).

The proposal that formation of a supercoiled loop is important in Mu replication, and that loop formation is controlled from the apex rather than from the base, may prove useful in understanding the organization of bacterial chromosomes. If rare, strong gyrase binding sites are present in the *E. coli* chromosome, as suggested by others (1a, 27, 36), they may assist in the formation of independently supercoiled chromosomal domains (6, 26, 35). Such chromosomal sites may be able to replace the SGS in the center of the Mu genome and stimulate phage replication. We are presently testing this possibility.

ACKNOWLEDGMENTS

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