JOURNAL OF BACTERIOLOGY, Aug. 1994, p. 5093–5100
0021-9193/94/$04.00+0

Vol. 176, No. 16

Chi-Dependent Formation of Linear Plasmid DNA in Exonuclease-Deficient *recBCD*$^+$ Strains of *Escherichia coli*

MUHAMMAD M. ZAMAN AND T. CHRISTIAN BOLES*

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Received 7 March 1994/Accepted 11 June 1994

***Escherichia coli* strains carrying mutations in *sbcB* (exonuclease I) or *xthA* (exonuclease III) accumulate high-molecular-weight linear plasmid concatemers when transformed with plasmids containing the chi sequence, 5′-GCTGGTGG-3′. Chi-dependent formation of high-molecular-weight plasmid DNA is dependent on *recA* and *recF* functions. In addition, chi stimulation occurs only in *cis*. Our data are consistent with models in which RecA and RecF proteins bind to and protect the DNA ends produced by RecBCD-chi interaction.**

The RecBCD enzyme of *Escherichia coli* is required by the major recombination pathway used during conjugation and generalized transduction (6, 39). RecBCD is also the principal enzymatic activity responsible for degradation of linear DNA molecules in vivo (38, 46). In vitro, purified RecBCD binds to the ends of linear double-stranded DNA molecules with flush (or nearly flush) ends and degrades both strands by using ATP-dependent helicase and double-strand exonuclease activities (see reference 42 for review). The enzyme has no activity on closed or nicked circular substrates (15, 19).

The paradox presented by RecBCD's seemingly conflicting roles in degradation and recombination has been partially explained by genetic and biochemical work showing that RecBCD is modulated by a specific DNA sequence called chi, 5′-GCTGGTGG-3′ (41). Efficient host-mediated recombination in phage lambda crosses depends on the presence of a chi sequence in at least one recombining chromosome (23). Chi-mediated stimulation of recombination is also dependent on the orientation of the chi sequence. Productive interactions require that RecBCD approaches a chi site from the 3′ side of the sequence, as stated above (20). Recombination is stimulated only in the interval to the 5′ side of chi, and the stimulation decreases with increasing distance from chi (4, 12).

In vitro studies have demonstrated chi-mediated alterations of RecBCD activities. Dixon and Kowalczykowski demonstrated that chi causes an attenuation of the enzyme's exonuclease activity without eliminating its helicase activity (10, 11). The single-stranded DNA ends which are produced by the continuing helicase reaction after chi recognition can initiate RecA-mediated strand exchange (10). Under different conditions, Taylor and Smith found that chi recognition causes attenuation of the exonuclease, helicase, and chi-specific endonuclease activities of RecBCD (43). On the basis of genetic studies, it has been proposed that chi recognition causes dissociation of the RecD subunit from the enzyme (35, 44), and recently some indirect biochemical evidence for this hypothesis has been obtained (9).

In vivo evidence for chi-mediated attenuation of RecBCD nuclease activity has been obtained by Dabert et al. (8), who found that broad-host-range plasmids that replicate by a rolling circle mechanism will produce high-molecular-weight (HMW) linear plasmid DNA in *E. coli* if the plasmid contains a chi sequence. The authors proposed that chi acts by preventing RecBCD-mediated degradation of rolling circle intermediates that escape normal replication termination.

We have sought to develop methods for analyzing the in vivo DNA intermediates of the RecBCD recombination pathway. As a first step toward this goal, we have tried to develop a plasmid system that recombines via the RecBCD pathway. Since plasmids are mostly in a closed circular form in vivo, plasmid recombination is normally carried out by a different pathway, the RecF pathway, which does not require RecBCD function (18, 22).

Our approach is based on a well-known property of *recBC* mutants that also carry mutations in the gene for exonuclease I, *sbcB*. In these *recBC sbcB* mutants, plasmids can switch from normal theta-style replication to a rolling circle mode which produces large amounts of HMW linear concatemeric DNA (7). This phenomenon suggests that elimination of rolling circle replication intermediates is one of the major roles of RecBCD in vivo.

In this paper, we report that plasmids containing chi sites form HMW linear plasmid concatemers in *sbcB* and *xthA* (exonuclease III) single mutants. We show that chi-mediated HMW plasmid formation in *sbcB* strains is dependent on *recA* and *recF* functions. In addition, the chi effect occurs only in *cis*. We discuss these results in terms of a model in which the *recA* and *recF* gene products cooperate to protect the DNA ends produced by RecBCD-chi interaction.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All transformation was carried out by a $CaCl_2$ procedure (36). Usually, strains were freshly transformed the day before each experiment. Occasionally, transformants were kept on selective plates at 4°C for up to 1 week prior to use. Transformants were selected on LB medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) supplemented with ampicillin (100 μg/ml for plates, 50 μg/ml for liquid media) or spectinomycin (50 μg/ml for plates and liquid media). All cultures were grown at 37°C.

Plasmids. All plasmids used in this study were derivatives of pBR322 (3) or pCL1920 (26). pBR322 is known to lack chi sites (40). Except for a 243-bp region near the 5′ end of the spectinomycin resistance gene, the sequence of pCL1920 can be deduced from the literature (2, 5, 17, 32, 48), and that sequence also lacks chi sites (data not shown).

pMMZ15 was constructed by inserting a chi-containing

* Corresponding author. Present address: 5 Judith Lane, no. 6, Waltham, MA 02154. Electronic mail address: cboles@world.std.com.

TABLE 1. *E. coli* strains

Strain	Relevant genotype	Additional mutations present	Source
AB1157	*rec*$^+$	*argE3 his-4 leu-6 proA2 thr-1 thi-1 rpsL31 galK2 lacY1 ara-14 xyl-5 mtl-1 supE44 kdgK51*	A. J. Clark
RDK1899	*sbcB15*	As for AB1157	S. T. Lovett
JC7623[a]	*sbcB15 recB15 recC22*	As for AB1157	A. J. Clark
JC8814	*sbcB15 recF143*	As for AB1157	S. T. Lovett
STL1671	*sbcB15* del(*srlR-recA*)*304*	As for AB1157	S. T. Lovett
SK1314	*xthA1*	*argE3 his-4 thi-1* Spc^r *rpsE lacMS286* φ80dII *lacBK1*	S. R. Kushner
SK4642	del(*sbcB*)	*lac-301 argE3* del(*his*) *leu307*(Am) *trpE9829*(Am) *rpsL321* Tet^r *supD*	S. R. Kushner
JC10287	del(*srlR-recA*)*304*	As for AB1157	A. J. Clark

[a] This strain is also likely to carry *sbcC201* (27).

double-stranded oligonucleotide into the *Bam*HI site of pBR322. The double-stranded oligonucleotide was formed by annealing 5'-GATCCTCTAGAGCTGGTGGG-3' and 5'-GATCCCCACCAGCTCTAGAG-3'. The oligonucleotide also contains an *Xba*I site, which was used to screen for chi$^+$ constructs. In pMMZ15, the chi sequence reads 5'-GCTGGTGG-3' in the clockwise orientation for the map shown in Fig. 1. pMMZ16 was constructed in the same way as pMMZ15, except that the chi site is in the opposite orientation.

pMMZ18 (chi^0) was constructed by ligating the large *Hin*dIII-*Sal*I fragment of pCL1920 to the 631-bp *Hin*dIII-*Sal*I fragment of pBR322. pMMZ19 (chi$^+$) was constructed by ligating the large *Hin*dIII-*Sal*I fragment of pCL1920 to the 651-bp chi$^+$ *Hin*dIII-*Sal*I fragment of pMMZ15. All constructs were amplified and stored in the del(*recA*) strain JC10287.

In preliminary studies, pBR322 derivatives carrying a different chi-containing duplex oligonucleotide were used. The oligonucleotide (5'-CAGCTGGTGG-3') was cloned into the Klenow fragment-filled *Nhe*I site of pBR322 by blunt-end ligation.

Preparation of total DNA. Cells were grown to mid-log phase (about 2×10^8 to 3×10^8 cells per ml), pelleted by centrifugation at 12,000 × *g* for 5 min, and resuspended in lysis buffer (100 mM Tris-HCl [pH 7.6], 1% sodium dodecyl sulfate [SDS], 30 mM EDTA) containing 1 mg of proteinase K per ml. Two hundred microliters of lysis solution was used for each milliliter of culture. Proteinase K digestion was continued overnight at 25°C. After digestion, the DNA was partially sheared by pipetting the cell lysate 20 times through a 200-μl disposable pipette tip. After shearing, the DNA was extracted twice with phenol and ethanol precipitated.

Southern hybridization analysis of plasmid species. DNA samples were run in 1% agarose (Sigma) gels with TAE buffer (50 mM Tris-acetate [pH 7.8], 5 mM sodium acetate, 1 mM EDTA) for 18 h at 1.42 V/cm. DNA transfer to nylon filters (Nytran; Schleicher and Schuell) and blot hybridization with ^{32}P-labeled probes were carried out as described previously (36). Plasmid probes were produced by the random priming method (13, 14) with a commercially available kit (Boehringer Mannheim). For pBR322-specific probes, the 454-bp *Ase*I-*Xmn*I fragment from the ampicillin resistance gene was used. For pCL1920-specific probes, the 550-bp *Alw*NI-*Spe*I fragment was used.

Exonuclease digestion. Total DNA was simultaneously digested with exonuclease I (U.S. Biochemicals) and lambda exonuclease (Gibco BRL), under conditions that degrade linear DNA but not open or closed circular DNA. The reaction conditions were 67 mM glycine-KOH (pH 9.4), 2.5 mM $MgCl_2$, 50 μg of bovine serum albumin per ml, 5 μg of total DNA per ml, 50 U of lambda exonuclease per ml, and 50 U of exonuclease I per ml. Reactions were allowed to proceed at 37°C for 30 min. Reactions were terminated by extracting the DNA twice with phenol prior to ethanol precipitation.

RESULTS

Chi-dependent formation of HMW linear plasmid DNA in *recBCD*$^+$ strains. To test HMW plasmid formation, plasmids

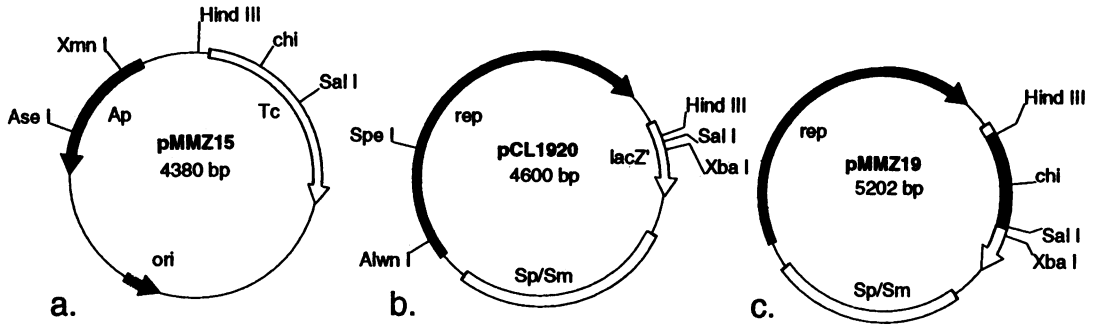

FIG. 1. Diagram of plasmids. (a) pMMZ15 is a chi$^+$ derivative of pBR322 carrying a chi-containing oligonucleotide inserted at the *Bam*HI site. The oligonucleotide also carries an *Xba*I site to facilitate screening. In pMMZ15, chi is read 5'-GCTGGTGG-3' clockwise on the map shown. In pMMZ16, the chi site is in the opposite orientation. (b) pCL1920 is based on the pSC101 replicon (rep) and carries spectinomycin resistance (Sp) (26). (c) pMMZ19 is a derivative of pCL1920 carrying the 651-bp chi$^+$ *Hin*dIII-*Sal*I fragment of pMMZ15 inserted between the *Hin*dIII and *Sal*I sites of pCL1920. pMMZ18 is the chi^0 version of pMMZ19. Ap, ampicillin resistance; Tc, tetracycline resistance; Sm, streptomycin resistance.

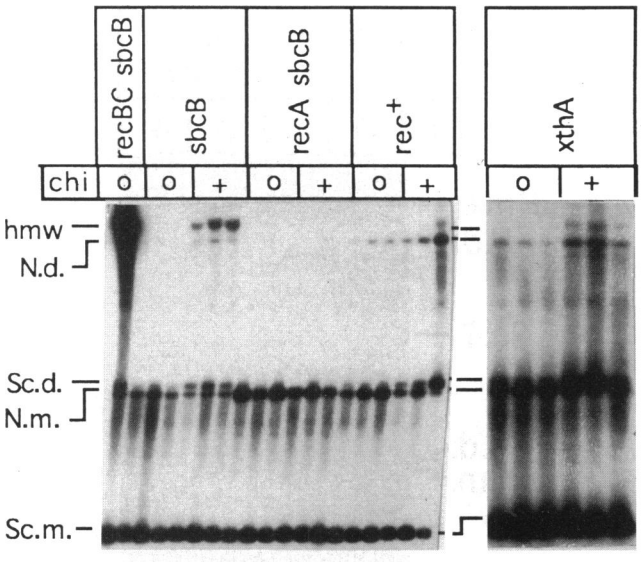

FIG. 2. Southern blot analysis of chi^0 and chi$^+$ plasmids in *sbcB* and *xthA* strains. Strains with the indicated genotypes were transformed with pBR322 (chi^0) or its chi$^+$ derivative, pMMZ15. Total DNA was prepared from mid-log cultures of three independent transformants of each strain. Samples were electrophoresed in 1% agarose gels, transferred to nylon filters, and probed with ^{32}P-labelled pBR322. The positions of supercoiled monomer (Sc.m.), nicked monomer (N.m.), supercoiled dimer (Sc.d.), nicked dimer (N.d.), and HMW (hmw) plasmid DNA were determined from markers which were visualized by ethidium bromide staining prior to blotting (data not shown). Strains used were JC7623 (*recBC sbcB*), RDK1899 (*sbcB*), STL1671 [del(*recA*) *sbcB*], AB1157 (*rec*$^+$), and SK1314 (*xthA1*).

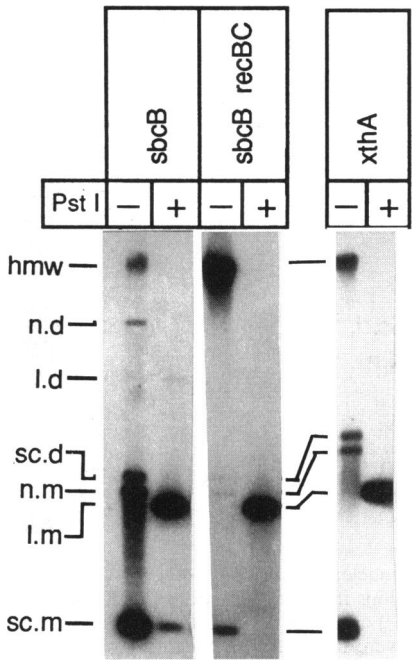

FIG. 3. Restriction analysis of HMW plasmid DNA. Total DNA preparations from chi$^+$ (pMMZ15) transformants were digested with *Pst*I and subjected to electrophoresis and blot hybridization with pBR322 probes as described in the legend to Fig. 2. The sizes of the hybridizing fragments were determined by using molecular weight standards visualized by ethidium bromide staining prior to blotting (data not shown). l.m. and l.d. indicate linear monomers and linear dimers, respectively. Other abbreviations are as described in the legend to Fig. 2.

with (chi$^+$) and without (chi^0) chi sites were transformed separately into *E. coli* strains. Individual transformants were grown to mid-log phase, and total DNA was prepared by lysis with SDS and proteinase K. DNA samples were electrophoresed in agarose gels and blotted to nylon filters, and plasmid DNA was detected by hybridization with ^{32}P-labeled plasmid probes and autoradiography. Under the electrophoresis conditions used, linear DNA greater than 20 kb in length migrates as a single low-mobility band, indicated in the figures by HMW. To identify this position in the gels, the *E. coli* chromosomal DNA, which is visible in ethidium-stained gels (data not shown), or samples of HMW pBR322 DNA prepared from a *recBC sbcB* strain (7) were used as markers. The chi$^+$ plasmids were constructed by cloning a synthetic oligonucleotide containing the chi sequence into the *Bam*HI site of pBR322, as illustrated in Fig. 1a. The chi sequence of pMMZ15 reads 5′-GCTGGTGG-3′ in the clockwise direction as drawn in Fig. 1a, whereas the chi sequence of pMMZ16 is in the opposite orientation.

In Fig. 2, data obtained from pBR322 (chi^0) and pMMZ15 (chi$^+$) transformants of several different strains are shown. When the *recBCD*$^+$ *sbcB*$^+$ strain AB1157 (*rec*$^+$ in Fig. 2) is used, HMW plasmid DNA is not formed in most transformants regardless of the chi genotype of the plasmid. In contrast, all chi$^+$ transformants of the *sbcB* strain form HMW plasmid DNA. No HMW plasmid DNA is seen in chi^0 transformants of the *sbcB* strain. Similar experiments with pMMZ16 (chi$^+$) gave identical results, demonstrating that the chi effect is independent of chi orientation. The fraction of chi$^+$ plasmid migrating as HMW DNA in *sbcB* strains is much lower than that observed in pBR322 samples isolated from *recBC sbcB* mutants (Fig. 2, left lanes).

To ensure that the results in Fig. 2 were attributable to the chi sequence and not to some other feature of the inserted oligonucleotide, the experiments were repeated with a pBR322 derivative carrying a different chi-containing oligonucleotide inserted at a different plasmid location (see Materials and Methods). The results obtained with this plasmid were identical to those in Fig. 2 (data not shown).

To verify that the HMW material is linear concatemeric plasmid DNA, we performed the restriction endonuclease and exonuclease digestions shown in Fig. 3 and 4. The concatemeric nature of the HMW plasmid DNA is demonstrated by the production of a single monomeric plasmid product after complete cleavage with a restriction enzyme which cuts the monomer plasmid only once (Fig. 3). The linear nature of the HMW DNA is confirmed by the experiments of Fig. 4, which show that the HMW material was completely digested by a mixture of exonuclease I and lambda exonuclease under conditions in which circular forms remain undegraded.

Chi-dependent HMW plasmid formation is not dependent on the specific *sbcB* allele used in these experiments, *sbcB15*, since identical results were obtained with the *sbcB* deletion strain SK4642 (data not shown). This result suggests that the formation of HMW plasmid DNA in the *sbcB* mutant is due to a deficiency of exonuclease I activity.

Since Niki et al. (30) reported that *recBC xthA* strains accumulate HMW plasmid DNA, we also tested *xthA* strains for chi-dependent HMW DNA formation. Our results for the *xthA* strain (Fig. 2, right lanes) are similar to those obtained for

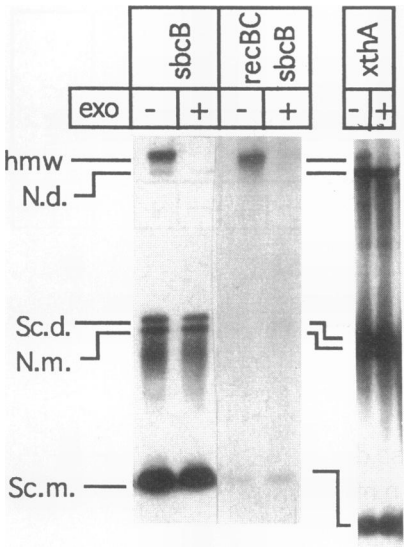

FIG. 4. Exonuclease sensitivity of HMW plasmid DNA. Total DNA preparations from an *sbcB* strain (RDK1899) transformed with pMMZ15 (chi$^+$) and from a *recBC sbcB* strain (JC7623) transformed with pBR322 were digested simultaneously with lambda exonuclease and exonuclease I as described in Materials and Methods. After digestion, the DNA was analyzed by electrophoresis and blot hybridized with pBR322 probes as described in the legend to Fig. 2. The abbreviations are as described in the legend to Fig. 2.

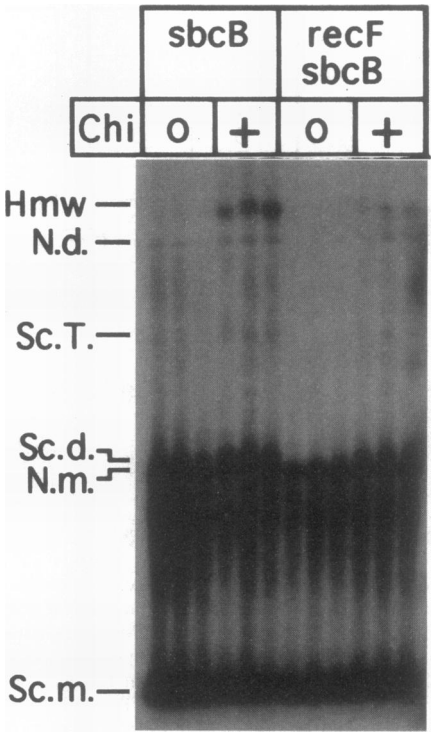

FIG. 5. Southern blot analysis of chi^0 and chi$^+$ plasmids in *sbcB* and *sbcB recF* strains. Strains with the indicated genotypes (RDK1899, JC8814) were transformed with pBR322 (chi^0) or its chi$^+$ derivative, pMMZ15. Total DNA was prepared from mid-log-phase cultures of three independent transformants of each strain and analyzed as described in the legend to Fig. 2. The abbreviations are as described in the legend to Fig. 2.

the *sbcB* strain, although the level of HMW plasmid DNA is lower in the *xthA* strains.

The role of recombination functions in chi-mediated HMW plasmid DNA formation. In previous work from other laboratories, HMW linear plasmid DNA formation was dependent on the RecF recombination pathway genes *recA*, *recF*, *recJ*, *recO*, and *recQ* (21, 37). To test the requirement for recombination function in chi-dependent HMW plasmid formation, a del(*recA*)*sbcB* strain was transformed with chi$^+$ and chi^0 plasmids and assayed as shown in Fig. 2. No HMW plasmid was observed in chi$^+$ or chi^0 transformants of this strain, indicating that *recA* function is required for HMW plasmid production in the *sbcB* background.

Similar experiments were performed with a *recF sbcB* strain to test the requirement of *recF* function in chi-mediated HMW plasmid formation. By visual inspection of Fig. 5, we estimate that the *recF* mutation decreases the amount of HMW plasmid DNA by at least fivefold. Thus, chi-mediated HMW plasmid formation shows a strong but not absolute dependence on *recF* function.

Testing a recombinational initiation role for chi in HMW plasmid formation. The results of the previous section suggest the possibility that chi may stimulate HMW plasmid formation by a mechanism which involves recombination. In the *recBC sbcB* system, two different models for the role of recombination in the formation of HMW plasmid DNA have been proposed. The first model holds that plasmid recombination creates replication forks that initiate rolling circle plasmid replication (7, 21, 37, 47). The second model proposes that intramolecular recombination reactions in the rolling circle tails create intermediate structures which prevent degradation by cellular exonucleases (7, 30). To investigate the relevance of these models to chi-mediated HMW plasmid synthesis, we tested the ability of chi sites to act in *trans*. The recombinational initiation model predicts that a chi$^+$ plasmid could activate HMW plasmid formation in a chi^0 plasmid in *trans* if the two plasmids share homology. The degradation model predicts that chi will stimulate HMW DNA formation only in *cis*.

Two partially homologous compatible plasmids were transformed into an *sbcB* strain. One of the two plasmids contained a chi site in the region of homology. Using probes specific for each plasmid, we analyzed DNA samples from the double transformants for HMW DNA derived from each plasmid. The results are shown in Fig. 6 and 7. The plasmids used are represented by a and b in Fig. 6 and 7 and are illustrated schematically in Fig. 1. The a plasmids are pBR322 and its chi$^+$ derivative, pMMZ15. The b plasmids are derivatives of pCL1920 (26). pCL1920 uses a pSC101 replicon and has no homology to pBR322. To create plasmids that have a limited region of homology with pBR322 and pMMZ15, the small *Hin*dIII-*Sal*I fragments from pBR322 and pMMZ15 were cloned into pCL1920 to create pMMZ18 (chi^0) and pMMZ19 (chi$^+$). Figures 6 and 7 show that the chi$^+$ plasmid always formed HMW plasmid DNA, whereas the partially homologous chi^0 plasmid did not. This result is not caused by selective loss of cells which contain HMW DNA from both plasmids, since this condition is readily observed when both plasmids carry chi sites (Fig. 6 and 7, lanes 10 to 13).

A small amount of chi^0 HMW plasmid DNA is observed in Fig. 6, lane 7, and Fig. 7, lanes 2 to 5. We examined these samples to determine whether the HMW chi^0 DNA was formed from rolling circle replication of the monomer chi^0 plasmid, representing authentic chi stimulation in *trans*, or

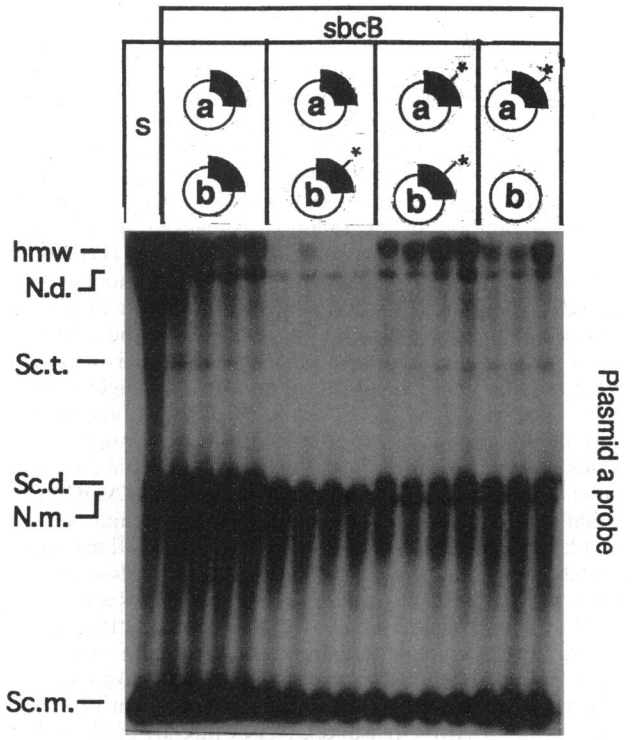

FIG. 6. Analysis of *cis* and *trans* activity of chi with a pBR322-specific probe. An *sbcB* strain (RDK1899) was cotransformed with derivatives of pBR322, plasmid a (colEI origin, Ap^r), and pCL1920, plasmid b (pSC101 origin, Sp^r), as depicted schematically at the top of the figure. The thickened segment indicates the 631-bp *Hin*dIII-*Sal*I fragment of pBR322. Fragments carrying chi sites (inserted at the pBR322 *Bam*HI site within the *Hin*dIII-*Sal*I fragment) are indicated by an asterisk. Total DNA samples were electrophoresed and blot hybridized with a pBR322-specific probe. Each lane contains DNA from an independent cotransformant. The plasmid combinations used were (from left to right) pMMZ15 and PMMZ18, pBR322 and pMMZ19, pMMZ15 and pMMZ19, and pMMZ15 and pCL1920. s represents sample HMW (hmw) pBR322 DNA prepared from a *recBC sbcB* strain (JC7623). Sc.m., supercoiled monomer; N.m., nicked monomer; Sc.d., supercoiled dimer; N.d., nicked dimer; S.c.t., supercoiled trimer.

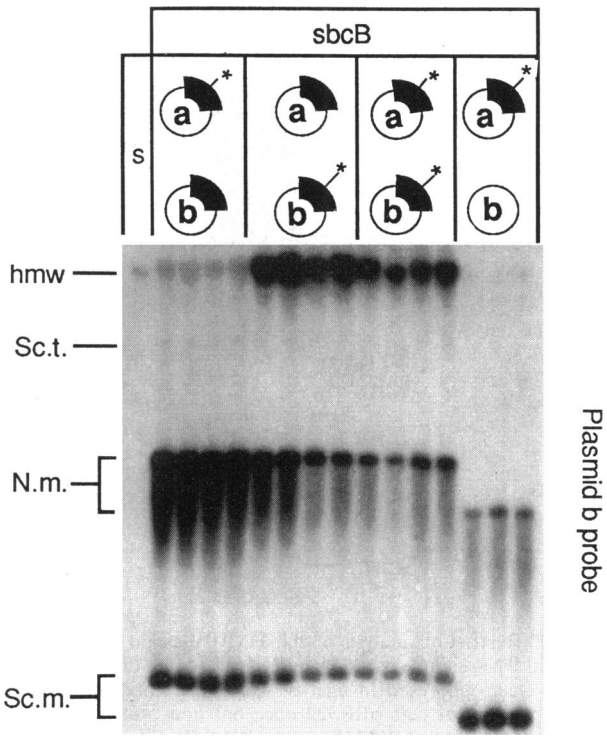

FIG. 7. Analysis of *cis* and *trans* activity of chi with a pCL1920 sequence-specific probe. The blot used for Fig. 6 was stripped and reprobed with a probe that detects only pCL1920 sequences. All abbreviations are described in the legend to Fig. 6.

whether the HMW chi^0 DNA was produced from a heterodimer containing both types of monomer. As shown in Fig. 8, we found that the HMW DNA in the sample shown in Fig. 6, lane 7, was derived from a heterodimer, since it is digested to the expected heterodimer size by enzymes which only cut one of the two monomer plasmids. Similar results were obtained for the HMW DNA in the samples of Fig. 7, lanes 2 to 5 (data not shown).

These results are most compatible with models in which the chi sites act in *cis* to cause formation of HMW plasmid DNA and therefore argue against models in which chi acts by recombinational initiation of plasmid rolling circle replication. However, these experiments do not rule out the possibility that chi-independent plasmid recombination may play a role during initiation of HMW plasmid DNA synthesis.

Stability of chi^+ plasmids in *sbcB* strains. *recBC sbcB* strains show high rates of plasmid loss under nonselective growth conditions (1, 33). This phenotype is correlated with a toxic effect of HMW plasmid accumulation on host cell growth (21). For this reason, we tested for chi effects on plasmid stability in the *sbcB* background with the method of Bassett and Kushner (1). Single transformants were grown in selective liquid medium to mid-log phase and then were plated on nonselective agar medium. One hundred of the resulting colonies were replicated to nonselective and selective plates to determine the number of colonies that had lost plasmids. Using this assay, we found that the chi^+ (pMMZ15, pMMZ16) and chi^0 (pBR322) plasmids were equally stable in *sbcB* strains: 99 to 100 of 100 nonselectively grown colonies retained the plasmid. With the same assay, pBR322 was completely unstable in a *recBC sbcB* mutant; none of the 100 nonselectively grown colonies retained the plasmid after growth on nonselective plates. Therefore, chi-dependent HMW DNA in *sbcB* strains differs in structure or amount from the HMW plasmid DNA in *recBC sbcB* strains in a way that dramatically reduces problems during plasmid segregation.

DISCUSSION

There are basically two ways in which chi sites could lead to the accumulation of HMW plasmid DNA. First, chi sites could act positively to initiate or increase production of HMW plasmid DNA. Second, chi could act negatively to suppress degradation of HMW plasmid DNA that had been formed in a chi-independent process. In Fig. 9, we consider models of these two classes that are consistent with our observation that the process is dependent on the *recA* and *recF* gene products (Fig. 2 and 5).

In Fig. 9a, chi sites act by stimulating intermolecular pairing events that initiate rolling circle replication and HMW plasmid DNA formation (7, 21, 27, 47). Contrary to the prediction of

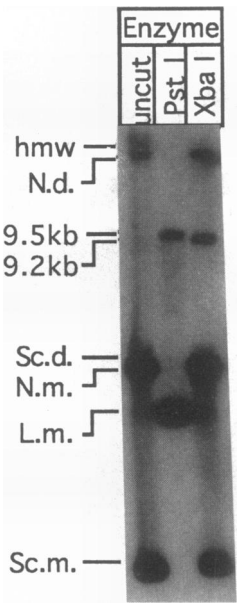

FIG. 8. Restriction analysis of chi^0 HMW plasmid DNA. Aliquots of the total DNA sample used in lane 7 of Fig. 6 were digested with *Pst*I or *Xba*I, electrophoresed in agarose gels, and blot hybridized with pBR322 probe. *Pst*I cuts pBR322 once but does not cut pMMZ19. *Xba*I cuts pMMZ19 twice (300 bp apart) but does not cut pBR322. *Pst*I digestion therefore produces a 4.3-kb fragment from pBR322 and a 9.5-kb fragment from a pBR322-pMMZ18 heterodimer. *Xba*I produces a 9.2-kb fragment from a pBR322-pMMZ19 heterodimer. L.m., linear monomer. Other abbreviations are as described in the legend to Fig. 6.

this model, the data presented in Fig. 6 and 7 demonstrate that chi usually, if not exclusively, acts in *cis* to stimulate HMW plasmid formation. Therefore, our data argue against a role for chi in initiation of HMW plasmid synthesis.

Our results do not exclude the possibility that chi-independent recombination is required for initiation of HMW plasmid synthesis, as proposed by others working with the *recBC sbcB* background (7, 21, 27, 47). The *recF* dependence of HMW plasmid synthesis in our system (Fig. 5) is consistent with this proposal, although other interpretations are discussed below.

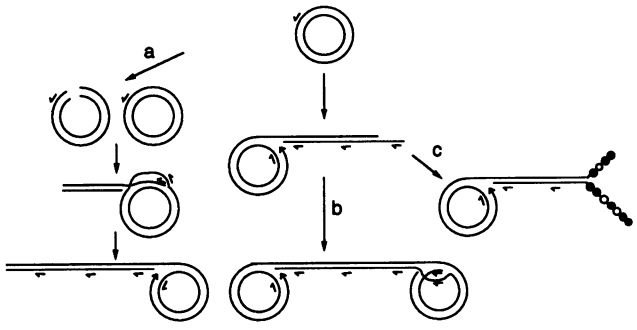

FIG. 9. Models for the role of chi in HMW plasmid formation in *sbcB* strains. In pathway a, chi sites on one plasmid stimulate recombination events that initiate rolling circle replication in another plasmid. In pathways b and c, chi sites interact directly with RecBCD enzyme to attenuate its exonuclease activity. In pathway b, the tails produced by RecBCD-chi interaction form intramolecular recombination intermediates that further inhibit degradation. In pathway c, additional protection of rolling circle forms is caused by direct binding of RecA (●) and RecF (○) to the single-stranded DNA ends produced by RecBCD helicase activity.

Two models in which chi acts negatively to prevent degradation of HMW plasmid DNA are shown in paths b and c of Fig. 9. In both cases, the primary function of chi is to prevent degradation by direct interaction with RecBCD enzyme. Additional inhibition of degradation is caused by secondary processes that prevent access of RecBCD to the HMW DNA in a *recA*- and *recF*-dependent fashion. In model b, the secondary inhibition is caused by the formation of terminal intramolecular recombination intermediates in the rolling circle tails. In model c, the secondary inhibition is caused by direct binding of RecA and RecF proteins to single-stranded DNA ends produced by RecBCD-chi interactions. Both of these inhibition models are consistent with the data presented in this paper. However, in other work (49), we have obtained evidence that indirectly argues in favor of path c. As described in that report, plasmid recombination in *sbcB* strains is greatly stimulated by plasmid chi sites. As expected, chi stimulation of recombination is dependent on *recA* function. However, it is independent of *recF* function. This suggests that RecF's role in HMW plasmid synthesis (Fig. 5) is not to stimulate the formation of recombination intermediates, as in model b of Fig. 9. Therefore, the *recF* effects appear to be most consistent with model c, in which RecA and RecF assist in preventing degradation of HMW plasmid DNA by directly binding to the DNA ends generated by RecBCD-chi interactions. A direct binding role for RecF is also consistent with genetic and biochemical evidence demonstrating that RecF is a single-stranded DNA-binding protein (16) that interacts with RecA protein (28, 29, 45).

It is important to note that for these simple models, chi cannot be acting in both ways, by stimulating recombinational initiation of rolling circle replication forks and also by slowing degradation of the linear tails. This is because the orientation of chi which would be required for chi-dependent recombinational initiation of rolling circle replication cannot produce linear tails in which chi sites are properly oriented to inhibit RecBCD degradation and vice versa (compare Fig. 9a and b).

The *recF* dependence of chi-mediated HMW plasmid synthesis suggests that RecF protein plays important roles in DNA metabolism in *recBCD*$^+$ strains. Previously, Lanzov et al. (24) found that *recF* mutations decrease the density of crossovers during conjugational recombination by the RecBCD pathway. Additional work will be required to establish how that observation is related to our results.

Our study confirms and extends the findings of Dabert et al. (8), who observed chi-dependent protection of HMW linear DNA formed from plasmids that replicate by a rolling circle mechanism. In their system, the chi effect was dependent on *recA* function, in agreement with our results, and they also proposed that RecA binding protects DNA ends generated by RecBCD-chi interaction. However, their study demonstrated that plasmid chi sites can act in *trans* to protect DNA from RecBCD-sensitive gene 2 mutants of phage T4. In contrast, no *trans* activity was found in our system (Fig. 6 and 7). The larger amount of HMW plasmid DNA produced in their system may account for this difference.

The similar effects of *sbcB* and *xthA* mutations on HMW plasmid formation are difficult to reconcile with the enzymatic differences between exonuclease I (25) and exonuclease III (34). These differences argue against models in which exonucleases I and III assist RecBCD enzyme in degrading HMW plasmid DNA. In addition, the work of Dabert et al. (8)

demonstrates that HMW plasmid formation induced by a rolling circle replicon occurs efficiently in *sbcB*$^+$ *xthA*$^+$ strains. In agreement with these results, we have found that dimeric chi$^+$ plasmids occasionally form HMW plasmid DNA in *recBCD*$^+$ *sbcB*$^+$ *xthA*$^+$ strains (Fig. 2, *rec*$^+$ strain, right lane). These observations are most consistent with a model in which exonucleases I and III normally suppress aberrant processes that lead to rolling circle plasmid replication. Thus, if rolling circle plasmid replication is initiated by a process that circumvents the steps inhibited by *sbcB*$^+$ or *xthA*$^+$, HMW plasmid DNA from chi$^+$ plasmids will be formed, even in *recBC*$^+$ *sbcB*$^+$ *xthA*$^+$ strains.

Our data suggest that intermediates of productive RecBCD-chi interactions are responsible for the accumulation of chi$^+$ HMW plasmid DNA and therefore should be present in the HMW fraction. If sufficiently gentle techniques for isolating the HMW plasmid DNA can be perfected, structural studies on the intermediates could provide new insights into the mechanisms of homologous recombination.

ACKNOWLEDGMENTS

We thank Tong Yang, Manisha Tiwari, and Jin Wang for performing important preliminary experiments; Susan Lovett, Sidney Kushner, and Richard Kolodner for generously providing bacterial strains; C. G. Lerner and M. Inouye for providing plasmid pCL1920; and Susan Lovett for numerous helpful discussions.

This work was supported by the Brandeis University Graduate Department of Biochemistry and by NIH BRSG grants to Brandeis University.

REFERENCES

1. **Bassett, C. L., and S. R. Kushner.** 1984. Exonucleases I, III, and V are required for stability of ColE1-related plasmids in *Escherichia coli*. J. Bacteriol. **157**:661–664.
2. **Bernardi, A., and F. Bernardi.** 1984. Complete sequence of pSC101. Nucleic Acids Res. **12**:9415–9426.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
4. **Cheng, K. C., and G. R. Smith.** 1989. Distribution of chi-stimulated recombination exchanges and heteroduplex endpoints in phage lambda. Genetics **123**:5–17.
5. **Churchward, G., D. Belin, and Y. Nagamine.** 1984. A pSC101-derived plasmid which shows no sequence homology to other commonly used cloning vectors. Gene **31**:165–171.
6. **Clark, A. J., and K. B. Low.** 1988. Pathways and systems of homologous recombination in *Escherichia coli* K-12. *In* K. B. Low (ed.), The recombination of genetic material. Academic Press, New York.
7. **Cohen, A., and A. J. Clark.** 1986. Synthesis of linear plasmid multimers in *Escherichia coli* K-12. J. Bacteriol. **167**:327–335.
8. **Dabert, P., S. D. Ehrlich, and A. Gruss.** 1992. Chi sequence protects against RecBCD degradation of DNA *in vivo*. Proc. Natl. Acad. Sci. USA **89**:12073–12077.
9. **Dixon, D. A., J. J. Churchill, and S. C. Kowalczykowski.** 1994. Reversible inactivation of the *Escherichia coli* RecBCD enzyme by the recombination hotspot chi *in vitro*: evidence for functional inactivation or loss of the RecD subunit. Proc. Natl. Acad. Sci. USA **91**:2980–2984.
10. **Dixon, D. A., and S. C. Kowalczykowski.** 1991. Homologous pairing *in vitro* stimulated by the recombination hotspot, chi. Cell **66**:361–371.
11. **Dixon, D. A., and S. C. Kowalczykowski.** 1993. The recombination hotspot chi is a regulatory sequence that acts by attenuating the nuclease activity of the *E. coli* RecBCD enzyme. Cell **73**:87–96.
12. **Ennis, D. G., S. K. Amundsen, and G. R. Smith.** 1987. Genetic functions promoting homologous recombination in *Escherichia coli*: a study of inversions in phage lambda. Genetics **115**:11–24.
13. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
14. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–267. (Addendum.)
15. **Goldmark, P. J., and S. Linn.** 1972. Purification and properties of the *recBC* DNAse of *Escherichia coli* K-12. J. Biol. Chem. **247**: 1849–1860.
16. **Griffin, T. J., IV, and R. D. Kolodner.** 1990. Purification and preliminary characterization of the *Escherichia coli* K-12 RecF protein. J. Bacteriol. **172**:6291–6299.
17. **Hollingshead, S., and D. Vapnek.** 1985. Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenyltransferase. Plasmid **13**:17–30.
18. **James, A. A., P. T. Morrison, and R. D. Kolodner.** 1982. Genetic recombination of bacterial plasmid DNA: analysis of the effect of recombination-deficient mutations on plasmid recombination. J. Mol. Biol. **160**:411–430.
19. **Karu, A. E., V. MacKay, P. J. Goldmark, and S. Linn.** 1972. The *recBC* deoxyribonuclease of *Escherichia coli* K-12. J. Biol. Chem. **248**:4874–4884.
20. **Kobayashi, I., H. Murialdo, J. M. Crasemann, M. M. Stahl, and F. W. Stahl.** 1982. Orientation of cohesive end site cos determines the active orientation of c sequence in stimulating *recA-recBC*-mediated recombination in phage lambda lytic infections. Proc. Natl. Acad. Sci. USA **79**:5981–5985.
21. **Kusano, K., K. Nakayama, and N. Nakayama.** 1989. Plasmid-mediated lethality and plasmid multimer formation in an *Escherichia coli recBC sbcBC* mutant. J. Mol. Biol. **209**:623–634.
22. **Laban, A., and A. Cohen.** 1981. Interplasmidic and intraplasmidic recombination in *Escherichia coli* K-12. Mol. Gen. Genet. **184**: 200–207.
23. **Lam, S. T., M. M. Stahl, K. D. McMilin, and F. W. Stahl.** 1974. Rec-mediated recombinational hot spot activity in bacteriophage lambda. Genetics **77**:425–433.
24. **Lanzov, V., I. Stepanova, and G. Vinogradskaja.** 1991. Genetic control of recombination exchange frequency in *Escherichia coli* K-12. Biochimie **73**:305–312.
25. **Lehman, I. R., and A. L. Nussbaum.** 1964. The deoxyribonucleases of *Escherichia coli*. V. On the specificity of exonuclease I (phosphodiesterase). J. Biol. Chem. **239**:2628–2636.
26. **Lerner, C. G., and M. Inouye.** 1990. Low copy number plasmids for regulated low-level expression of cloned genes in *Escherichia coli* with blue/white insert capability. Nucleic Acids Res. **18**:4631.
27. **Lloyd, R. G., and C. Buckman.** 1985. Identification and genetic analysis of *sbcC* mutations in commonly used *recBC sbcB* strains of *Escherichia coli* K-12. J. Bacteriol. **164**:836–844.
28. **Madiraju, M. V. V. S., and A. J. Clark.** 1991. Effect of RecF protein on reactions catalysed by RecA protein. Nucleic Acids Res. **19**:6295–6300.
29. **Madiraju, M. V. V. S., A. Templin, and A. J. Clark.** 1988. Properties of a mutant *recA*-encoded protein reveal a possible role for *Escherichia coli recF*-encoded protein in genetic recombination. Proc. Natl. Acad. Sci. USA **85**:6592–6596.
30. **Niki, H., T. Ogura, and S. Hiraga.** 1990. Linear multimer formation of plasmid DNA in *Escherichia coli hopE* (*recD*) mutants. Mol. Gen. Genet. **224**:1–9.
31. **Phillips, G. J., D. C. Prasher, and S. R. Kushner.** 1988. Physical and biochemical characterization of cloned *sbcB* and *xonA* mutations from *Escherichia coli* K-12. J. Bacteriol. **170**:2089–2094.
32. **Prentki, P., and H. M. Krisch.** 1982. A modified pBR322 vector with improved properties for the cloning, recovery, and sequencing of blunt-ended DNA fragments. Gene **17**:189–196.
33. **Ream, L. W., N. Y. Crisona, and A. J. Clark.** 1978. ColE1 plasmid stability in Exo$^-$ ExoV$^-$ strains of *Escherichia coli* K-12, p. 78–80. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
34. **Richardson, C. C., I. R. Lehman, and A. Kornberg.** 1964. A deoxyribonucleic acid phosphatase-exonuclease from *Escherichia coli*. II. Characterization of the exonuclease activity. J. Biol. Chem. **239**:251–258.
35. **Rosenberg, S. M., and P. J. Hastings.** 1991. The split-end model for homologous recombination at double-strand breaks and at

Chi. Biochimie **73**:385–397.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Silberstein, Z., and A. Cohen.** 1987. Synthesis of linear multimers of OriC and pBR322 derivatives in *Escherichia coli* K-12: role of recombination and replication functions. J. Bacteriol. **169**:3131–3137.
38. **Simmon, V. F., and S. Lederberg.** 1972. Degradation of bacteriophage lambda deoxyribonucleic acid after restriction by *Escherichia coli* K-12. J. Bacteriol. **112**:161–169.
39. **Smith, G. R.** 1988. Homologous recombination in procaryotes. Microbiol. Rev. **52**:1–28.
40. **Smith, G. R., S. M. Kunes, D. W. Schultz, A. F. Taylor, and K. L. Truman.** 1981. Structure of Chi hotspots of generalized recombination. Cell **24**:429–436.
41. **Stahl, F. W.** 1986. Roles of double-strand breaks in generalized genetic recombination. Prog. Nucleic Acid Res. Mol. Biol. **33**:169–194.
42. **Taylor, A. F.** 1988. RecBCD enzyme of *Escherichia coli*, p. 231–263. *In* R. Kucherlapati and G. R. Smith (ed.), Genetic recombination. American Society for Microbiology, Washington, D.C.
43. **Taylor, A. F., and G. R. Smith.** 1992. RecBCD enzyme is altered upon cutting DNA at a Chi recombination hotspot. Proc. Natl. Acad. Sci. USA **89**:5226–5230.
44. **Thaler, D. S., E. Sampson, I. Siddiqi, S. M. Rosenberg, L. C. Thomason, F. W. Stahl, and M. M. Stahl.** 1989. Recombination of bacteriophage lambda in *recD* mutants of *Escherichia coli*. Genome **31**:53–67.
45. **Volkert, M. R., and M. A. Hartke.** 1984. Suppression of *Escherichia coli recF* mutations by *recA*-linked *srfA* mutations. J. Bacteriol. **157**:498–506.
46. **Willetts, N. S., and A. J. Clark.** 1969. Characteristics of some multiply recombination-deficient strains of *Escherichia coli*. J. Bacteriol. **100**:231–239.
47. **Yamamoto, K., K. Kusano, N. K. Takahashi, H. Yoshikura, and I. Kobayashi.** 1992. Gene conversion in the *Escherichia coli* RecF pathway: a successive half-crossing-over model. Mol. Gen. Genet. **234**:1–13.
48. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
49. **Zaman, M. M., and T. C. Boles.** Unpublished data.