Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 1624–1628, March 1987
Genetics

Large palindromes in the λ phage genome are preserved in a *rec*$^+$ host by inhibiting λ DNA replication

(recBC/sbcB/dnaBts/Escherichia coli)

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Communicated by Ira Herskowitz, November 13, 1986 (received for review June 25, 1986)

ABSTRACT **A large palindrome carried by phage λ has been shown to prevent growth of the phage on a *rec*$^+$ strain of *Escherichia coli*. The phage do form plaques on *recBC sbcB* strains, but the palindrome is not stable—deletions that either destroy the palindrome or diminish its size overgrow the original engineered palindrome-containing phage. We have prepared stocks of λ carrying a palindrome that is 2 × 4200 base pairs long. These phage stocks are produced by induction of a lysogen in which the two halves of the palindrome are stored at opposite ends of the prophage and are of sufficient titer (10^9 phage per ml) to enable one-step growth experiments with replication-blocked phage. We find that the large palindrome as well as a lesser palindrome of 2 × 265 base pairs are recovered intact among particles carrying unreplicated chromosomes following such an infection of a *rec*$^+$ host. We propose that DNA replication drives the extrusion of palindromic sequences *in vivo*, forming secondary structures that are substrates for the *recBC* and *sbcB* gene products.**

The literature is sprinkled with reports of the inability to clone or maintain DNA sequences containing large regions of palindromic symmetry in *Escherichia coli*. In some cases the palindrome was lethal to the vector (1–3); in other cases, *recA*-independent deletions occurred that removed the symmetry of the palindrome (4–6). Leach and Stahl (7) reported that λ carrying a 3200-base-pair (bp) palindrome could form plaques on a *recBC sbcB* strain of *E. coli* but not on a *rec*$^+$ strain. The phage was viable, but the palindrome was initially unstable in the *recBC sbcB* host. The palindrome was stable, however, following symmetrical or near symmetrical shrinkage to a size of 600 ± 100 bp. Phage carrying this smaller palindrome remained inviable on the *rec*$^+$ host. These results implicated the products of the *recBC* and *sbcB* genes, the recombination-related nucleases *recBC* gene protein and exonuclease I, in the destruction of palindrome-containing DNA.

Leach and Stahl (7) proposed that palindromes *in vivo* might adopt a cruciform structure, which is topologically equivalent to a recombination intermediate, the Holliday junction, and that this structure might be cleaved by *recBC* gene protein and exonuclease I. It has been argued that cruciforms do not form *in vivo* from palindrome sequences due to kinetic barriers (8) and attempts to detect cruciforms *in vivo* using short artificial palindromes cloned in plasmids have been unsuccessful (8, 9). These considerations suggest that *in vivo* cruciform formation is not an inherent property of palindromes. However, the kinetic arguments, based on studies carried out *in vitro* with purified DNA, may not be applicable *in vivo*, and cruciforms might be detected under conditions other than those tested thus far.

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We describe here the construction of a λ phage carrying a palindrome of 8400 ± 100 bp and show that the inviability and instability associated with both this palindrome and a 530-bp palindrome are dependent on replication of the phage DNA. The results of our studies imply that the activity of the DNA replication machinery facilitates the formation of nuclease-sensitive secondary structures from palindrome sequences.

MATERIALS AND METHODS

Phage and Bacterial Strains. λ phage used are described in Fig. 1. The *E. coli* K12 strains used are listed in Table 1.

Construction of FS1596. JC7623 (A. J. Clark, University of California, Berkeley), which is *recB21 recC22 sbcB15 lacY1 str31*, was transduced to *hflA1* with the aid of Tn*5* in a linked *pur* gene (in strain SB41 from D. Hagen, University of Oregon, Eugene). The resulting strain was transduced to *pur*$^+$, to eliminate Tn*5*, with P1 grown on the *hflA pur*$^+$ strain SB38 (D. Hagen). The resulting strain (FS1537) was P1-transduced to *hflB* with the aid of a linked Tn*10* (12). JC7623 gives clear plaques with wild-type λ; the *hflA* derivative (FS1537) gives turbid plaques; the *hflA hflB* derivative (FS1552) fails to allow wild-type λ growth. FS1552 was transduced to *lac*$^+$. The resulting F$^-$ strain (FS1578) was mated to HfrC strain KL752 (K. B. Low, Yale University, New Haven, CN), which is *lacZ813 lacI3 str*$^+$. A Lac$^-$ Str^R colony was designated FS1596. FS1596 does not allow λ*c*$^+$ growth, confirming *hflA hflB*. It does allow λ*c*I carrying a large palindrome to grow, confirming *recB* and/or *recC* plus *sbcB*. It is likely that the strain is both *recB* and *recC* and also *lacI3* and is so designated in Table 1. FS1596 is a derivative of AB1157 (13).

Density Gradient Experiments. λ phage were density labeled by UV induction of lysogens grown in minimal medium containing [^{13}C]glucose and $^{15}NH_4Cl$.

λ DNA synthesis was blocked, but transcription, recombination, and packaging were permitted, by infecting a *dnaBts* strain (FA77) at 42°C. FA77 was grown in broth at 26°C to a titer of 1.5 × 10^8 cells per ml, then shifted to 42°C for 10 min prior to infection with an equal volume of prewarmed phage at a titer of 1.5 × 10^9 plaque-forming units/ml. The cultures were supplemented with each of the four deoxynucleosides at 0.05 μg/ml and with glucose at 10 mg/ml to dilute out unincorporated isotope in the labeled stocks. The infected cells were aerated for 65 min at 42°C, then sedimented and resuspended to eliminate unadsorbed phage. Finally, the cells were lysed with $CHCl_3$ and eggwhite lysozyme. Experiments in which DNA synthesis was allowed were carried out as above except that a *dnaB*$^+$ strain (594) was used, and the temperature was maintained at 37°C throughout. The lysates obtained were brought up in cesium formate solution to a refractive index of 1.3775. Fractions were collected through the bottom of the tube after centrif-

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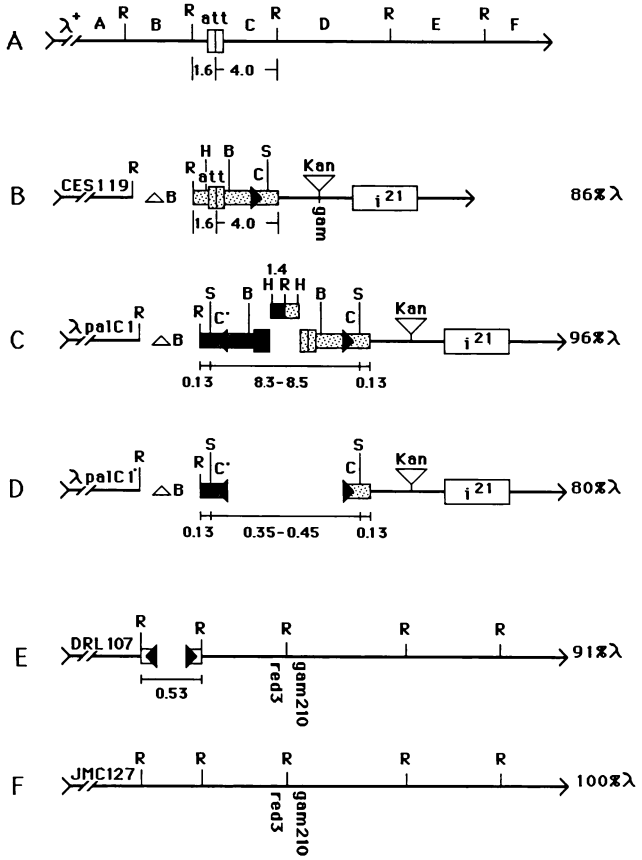

FIG. 1. Phage strains employed. (*A*) *Eco*RI restriction map of phage λ (not to scale). The *Eco*RI restriction sites (R) are designated by short vertical bars and divide λ into fragments A to F. The phage attachment site (*att*) divides fragment C into two parts of 1.6 kilobases and 4.0 kilobases, respectively. (*B–F*) Useful markers and restriction sites of λ strains used (not to scale). *Eco*RI sites present are designated R. Relevant *Bam*HI sites are designated B; *Hin*dIII sites, H; and *Sma* I sites, S. Distances are given in kilobases. Brackets denote deleted sequences. Kanamycin resistance (Kan^R) is specified by a transposase-deficient Kan^R derivative of Tn*10* inserted in the *gam* gene. i21 is the immunity region of phage 21. Arrowheads denote elements of inverted repeats. *red3* is unconditional; *gam210* is an amber mutation.

ugation for 20–24 hr at 32,000 rpm in a Beckman SW50.1 rotor. Each fraction was titrated on rec^+ and *recBC sbcB* indicator bacteria (JC9937 and JC9387, respectively).

RESULTS

Construction of λ Carrying an 8400-bp Palindrome. *In vitro* techniques were used to construct λ DNA molecules carrying duplications of λ fragment C (see Fig. 2 *A* and *B*), and λ DNA was packaged *in vitro*. To maximize preservation of palindromes, the packaged phage were allowed to infect FS1596, a *recBC sbcB* strain (7). FS1596 is *hflA hflB* to promote lysogenization at low multiplicities of infection. The cells were plated on Luria broth agar containing kanamycin at 50 μg/ml to select lysogens carrying phage with the right arm of parent II. The plates also contained 5-bromo-4-chloro-3-indolyl β-D-galactoside at 40 μg/ml as a screen for phage that no longer had the left arm of parent II. The individual Lac^- Kan^R lysogens obtained were induced by UV irradiation, and the resulting lysates were titrated on JC9387 (*recBC sbcB*) and JC9937 (rec^+). Three classes of lysogens were identified. One class produced phage that grew equally well on both indicators suggesting that they arose from clones carrying a single fragment C or a direct repeat of fragment C; the second class did not produce any phage particles, despite displaying immunity; and the third class produced phage that plated only on the *recBC sbcB* indicator, which was the phenotype expected for λ carrying a large inverted repeat. Phage DNA was prepared from five separate examples of this third class of lysogen and examined by restriction enzyme digestion and gel electrophoresis. The restriction pattern of the phage designated λpalC1 is shown in Fig. 1*C*; the other phage examined were indistinguishable from this. Although each phage carried a palindrome of some 8400 ± 100 bp, none carried an inverted repeat of the entire fragment C; the central *Eco*RI site and 1400–1500 bp of adjacent DNA were missing in every case. An explanation for this can be found by considering the structure of the prophage. Fragment C of λ carries the phage attachment site and is split into two parts during integration into the *E. coli* chromosome. Integration of a phage carrying an inverted repeat fragment C, whichever of the two possible attachment sites was used, would give rise to a prophage carrying a smaller 3200-bp palindrome of which one repeat was part of an intact fragment C (Fig. 2 *C* and *D*). When Southern hybridizations were carried out between total chromosomal DNA digested with *Eco*RI, or *Eco*RI and *Sma* I, and ^{32}P-labeled fragment C DNA, none of the prophage that gave rise to palindrome phage was found to contain an intact fragment C (data not shown). This suggests

Table 1. Bacterial strains

Strain	Relevant properties	Source or ref.
FS1596	*recB21 recC22 sbcB15 hflA hflB lacZ813 lacI3*	This paper
JC9387	*recB21 recC22 sbcB15* Su^-	A. J. Clark
JC9937	rec^+ Su^-, isogenic to JC9387	A. J. Clark
FA77	*dnaBts* Su^-	10
594	rec^+ Su^-	11

Su^-, nonsuppressing.

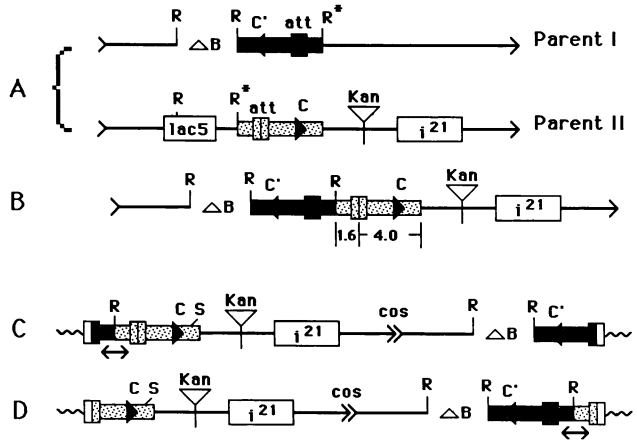

FIG. 2. Initial step in the isolation of λpalC1. Symbols are as in Fig. 1. (*A*) Parental phages, whose *Eco*RI fragments C are in opposite orientation (striped and stippled, respectively), were digested with *Eco*RI. (*B*) The digested parents were ligated with T4 ligase and packaged *in vitro* (14). Among the various products is one carrying an inverted repeat of the *Eco*RI fragment C, resulting from digesting and ligating of the *Eco*RI sites marked R* in *A*. (It is possible that λpalC1 carries the left-most *Eco*RI fragment from parent I rather than parent II. If so, it contains a bit of the *lac* fragment.) (*C* and *D*) The prophage that results from integration of the phage in *B* if the attachment site used were *attC'* (*C*) or *attC* (*D*). S denotes the *Sma* I site used to help define fragment C in the absence of its right-hand *Eco*RI site. The double-head arrows at the left (*C*) and right (*D*) ends of the prophage diagrams indicate the palindrome of 2 × 1.6 kilobases whose observed absence suggests that palindromes of such size are unstable in the *E. coli* chromosome of even a "permissive", *recBC sbcB*, strain.

selection against large intrachromosomal palindromes in *recBC sbcB E. coli*, implying that the 3200-bp palindrome was deleted *in situ*. If the deletion event extended into the prophage attachment site sequences, phage excision would be prevented, explaining the class of lysogens that did not produce phage on UV induction. The palindrome in λpalC1 must have been generated when those fragment C sequences that were not part of a palindrome in the prophage were brought into juxtaposition upon prophage excision. We have not established the exact endpoints of the intrachromosomal deletion in the prophage that gave rise to λpalC1, so we cannot say that λpalC1 carries a perfect inverted repeat. The restriction analysis of λpalC1 indicates that the extent of nonrepeated DNA is less than 150 bp.

Limited Stability of the Large Palindrome in a *recBC sbcB* Host. λpalC1 directly obtained from UV induction of the prophage was observed to form only tiny plaques on a *recBC sbcB* host, but after repeated cycles of lytic growth on that host an increase in plaque size was observed, and the majority of particles were now able to grow on the *rec*$^+$ indicator bacteria. These observations suggested that the palindrome was unstable even on the permissive host. Approximately 10% of the large plaque-forming phage remained unable to grow on the *rec*$^+$ indicator. Restriction analysis of several such phage revealed that the original palindrome was no longer present; instead the phage carried a smaller palindrome of 700 ± 100 bp. The 3200-bp palindrome of Leach and Stahl (7) also shrank, to a similar size, on infection of a *recBC sbcB* host. This shrinkage suggests that under conditions of lytic λ growth there is a maximum size limit for stability of palindromes in *recBC sbcB* strains of *E. coli*.

It has been demonstrated that deletions can occur that are approximately symmetrical about the center of palindromes (5, 6). The restriction patterns of λpalC1′, the derivative of λpalC1 that carries a shorter palindrome, and DRL107, the 530-bp palindrome-carrying phage of Leach and Stahl (7), are both consistent with an event of this type having occurred (see Fig. 1).

Replication of Palindrome Phage in a *rec*$^+$ Host. The model proposed by Leach and Stahl (7) to explain the inviability of λ carrying a perfect palindrome in a *rec*$^+$ host suggested that *recBC* gene protein and exonuclease I act directly on, or facilitate formation of, secondary structures derived from palindrome sequences. We can envisage several stages during the growth of λ where secondary structure formation could occur: (*i*) shortly after infection, when λ DNA circularizes and becomes negatively supercoiled, a state that is known to favor extrusion of palindromes *in vitro* (3, 15); (*ii*) during replication, stimulated by the unwinding of the duplex ahead of the replication fork; or (*iii*) during packaging of the phage DNA, where terminase cutting at *cos* permits *recBC* gene protein to enter and travel through the λ chromosome creating local regions of single-stranded DNA (16–18). We investigated the second of these possibilities by infecting *rec*$^+$ cells with density-labeled palindrome phage under conditions of free replication or with a replication block achieved by shifting a *dnaBts* strain to 42°C. The progeny phage were recovered after one cycle of growth and separated according to their density by centrifugation in cesium formate. The total number of phage in each fraction of the gradient was determined by plating on the *recBC sbcB* indicator (JC9387). The number of phage that could plate on the *rec*$^+$ indicator (JC9937) was also determined for each fraction. The results are shown in Figs. 3 and 4. Figs. 3*A* and 4*A* show the density profiles of control phages CES119 and JMC127, respectively, recovered from the replication-blocked infection. Peak I represents unreplicated (fully heavy) phage, as confirmed by the relative position of the density reference peak(s). The control phage plate equally well on both the *recBC sbcB* and the *rec*$^+$ indicators. Figs. 3*C* and 4*C* show the density profiles

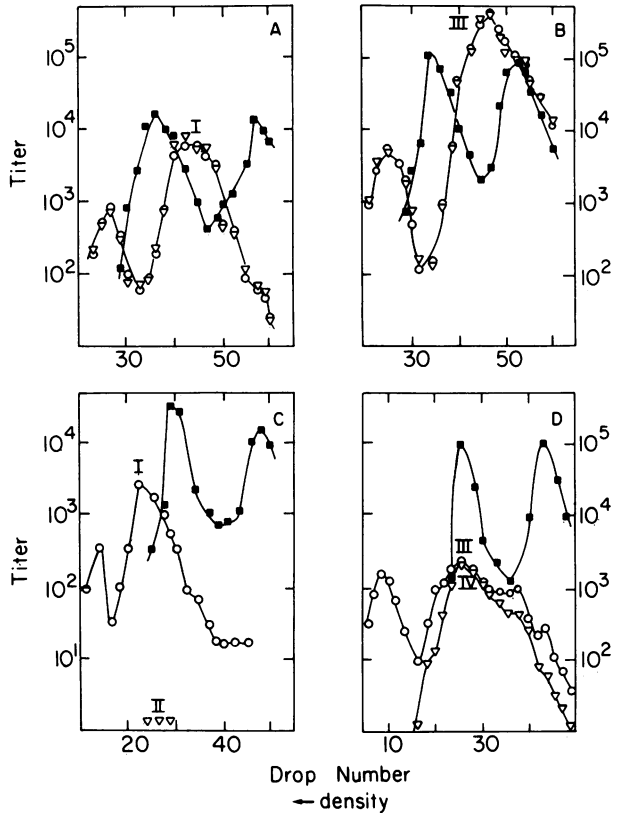

FIG. 3. Density distributions of λpalC1 and control phage grown in *rec*$^+$ hosts. ○, Total phage recovered, as titrated on the *recBC sbcB* strain JC9387. ▽, Phage able to grow on *rec*$^+$ strain JC9937. ■, Density references marking the approximate positions expected for fully light wild-type λ (left-hand peak) and fully light λ carrying a net deletion of 16% of the λ chromosome (right-hand peak). Phage were recovered from infections of the following types: (*A*) FA77 (*dnaB*ts) at 42°C with density-labeled control phage CES119 (see Fig. 1*B*). (*B*) 594 at 37°C with density-labeled CES119. CES119 carries a net deletion of 14% of the λ chromosome. (*C*) FA77 at 42°C with density-labeled λpalC1. (*D*) 594 at 37°C with density-labeled λpalC1. λpalC1 carries a net deletion of 4% of the λ chromosome. The left-most peak in each gradient represents unadsorbed phage.

of the palindrome phage λpalC1 and DRL107, respectively, recovered from the replication-blocked infection. Peak I represents the total yield of unreplicated phage, as titered on the *recBC sbcB* indicator. In both cases, the total yield of palindrome phage is approximately equal to the yield of control phage. Peak II represents phage that grew on the *rec*$^+$ indicator. The proportion of the total yield represented by these phage is the same as that found in the original phage stocks (≈5 in 10^3 phage for λpalC1 and ≈1 in 10^4 phage for DRL107) indicating that there was no loss of palindrome symmetry during the replication-blocked infection. Furthermore, λpalC1 did not undergo symmetrical shrinkage; peak I in Fig. 3*C* is located where expected, to the left of the density reference marker corresponding to wild-type (undeleted) λ. In the absence of replication, wild-type λ DNA molecules entering a cell will circularize, transcribe their genomes, and recombine to form dimeric circles that are substrates for packaging into mature phage particles. The recovery of phage in our experiment suggests that these same events can be carried out successfully by palindrome phage in the presence of *recBC* gene protein and exonuclease I. Furthermore, the 8400-bp and 530-bp palindromes are apparently maintained intact when replication is prevented.

When replication was allowed, palindrome phage that had undergone replication (Figs. 3*D* and 4*D*) were recovered in greatly reduced numbers compared with the control phage

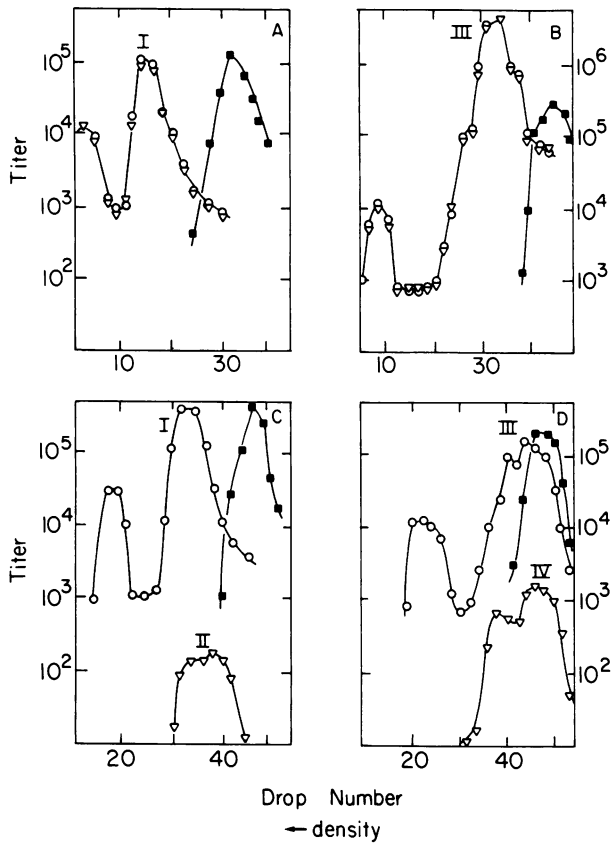

FIG. 4. Density distributions of DRL107 and control phage grown in *rec*$^+$ hosts. ○, Total phage recovered, as titrated on JC9387 (*recBC sbcB*). ▽, Phage able to grow on JC9937 (*rec*$^+$). ■, Density reference marking the approximate position expected for fully light λ carrying a net deletion of 10% of the λ chromosome. Phage were recovered from infections of the following types: (*A*) FA77 (*dnaB*ts) at 42°C with density-labeled control phage JMC127 (see Fig. 1*F*). (*B*) 594 at 37°C with density-labeled JMC127. JMC127 has the density of wild-type λ (*C*) FA77 at 42°C with density-labeled DRL107. (*D*) 594 at 37°C with density labeled DRL107. DRL107 carries a net deletion of 9% of the λ chromosome. The leftmost peak in each gradient represents unadsorbed phage.

(Figs. 3*B* and 4*B*). Peak III represents replicated phage as demonstrated by a shift in density toward the lighter side of the gradient. The yield of DRL107 (Fig. 4*D*) was lower by a factor of 20 than that of JMC127 (Fig. 4*B*), a figure that was confirmed by directly comparing the burst sizes of the two phage grown on the *rec*$^+$ host (Table 2), while the yield of λpalC1 (Fig. 3*D*) was lower by a factor of 250 than that of CES119 (Fig. 3*B*). Therefore, the presence of either palindrome was hazardous to phage undergoing DNA replication in the presence of *recBC* gene protein and exonuclease I, with the larger palindrome proving more deleterious than the smaller one.

Table 2. One-step growth experiments

Phage	Titer of progeny phage from one-step growth experiments	
	recBC sbcB host	*rec*$^+$ host
DRL107	5.23×10^8	3.8×10^6
JMC127	3.25×10^8	7.5×10^7

Bacteria (0.5 ml) at 1.5×10^8 cells per ml and 1.5×10^9 plaque-forming units/ml of phage were mixed and gently agitated at 34°C for 20 min. Buffer (5 ml) was added to each mixture, and the whole reaction mixture was filtered through a 0.45-μm Millipore filter to remove unadsorbed phage. Bacteria held on the filter were washed into 5 ml of tryptone broth. A suspension sample (2 ml) was treated with $CHCl_3$ and titrated on the *recBC sbcB* strain JC9387 to check for unadsorbed phage. Another suspension sample (3 ml) was aerated at 37°C for 60 min before lysing and titrating the progeny phage on the *recBC sbcB* strain.

The great majority of surviving λpalC1 phage, and 1 in 100 of the DRL107 phage recovered were able to grow on the *rec*$^+$ indicator. These phage are represented in peak IV of Figs. 3*D* and 4*D*. In the case of λpalC1 (Fig. 3*D*), peaks III and IV are closely aligned, indicating that most of the phage that replicated have disrupted palindromes. There are areas of divergence however, where the phage grow significantly better on the *recBC sbcB* indicator. The divergence on the left-hand (heavier) side of peak III can be assumed to represent palindrome phage that escaped replication. The divergence on the right-hand (lighter) side of peak III is probably due to phage that have deleted symmetrically and retained smaller palindromes. The phage that appear toward the right-hand side of the gradient have undergone extensive deletion and have densities approximating that of the reference marker carrying a 16% deletion of wild-type λ. λpalC1 itself carries a net deletion of only 4% of the λ chromosome.

Derivatives of DRL107 that had gained the ability to grow on the *rec*$^+$ indicator were isolated and subjected to restriction analysis (data not shown). Phage isolated from the right-hand (lighter) side of peak IV carried palindrome-disrupting deletions of widely varying size and end-point location. The larger deletions were found among the lightest phage particles, as would be expected. Phage isolated from the left-hand (heavier) side of the peak were found to carry insertions of unknown origin within the palindrome. In view of the variety of events observed, we are unable to invoke any single mechanism in the disruption of palindrome symmetry. Furthermore, although it is tempting to conclude from the density profiles that replication is instrumental in facilitating such disruption, the data do not allow us to distinguish this from selective replication of phage entering a *rec*$^+$ cell with a previously acquired deletion.

DISCUSSION

We have obtained λ phage, λpalC1, carrying a palindrome of 8400 ± 100 bp comprised of an inverted repeat of part of the λC fragment. λpalC1 is stored in a *recBC sbcB* host as a prophage in which most of the palindrome is split into two parts; excision of the prophage brings the two halves of the palindrome into juxtaposition. The phage can be prepared in high titer stocks by UV inducing the lysogen without harming the integrity of the palindrome. λpalC1 makes plaques on a *recBC sbcB* strain of *E. coli*, but not on a *rec*$^+$ strain. However, the palindrome is unstable during lytic growth within the permissive host. Among the progeny are particles that carry smaller palindromes. When the palindrome has shrunk to 700 ± 100 bp, it is stably maintained during lytic growth of the phage in a *recBC sbcB* host, but continues to confer inviability on the phage in a *rec*$^+$ host. The small number of phage obtained on a *rec*$^+$ host are due to particles containing disrupted palindromes. These findings are in agreement with those of Leach and Stahl (7) concerning λ phage carrying an inverted repeat of part of the λ fragment B and, therefore, appear to represent general characteristics of λ-borne palindromes in *E. coli*.

The model proposed by Leach and Stahl (7) to explain the lethal effects of palindromes invokes formation of secondary structures that are substrates for *recBC* gene protein and exonuclease I. We have shown here that palindrome-bearing phage fare as well as control phage if they do not undergo DNA replication, arguing that replication is responsible for the formation of nuclease-sensitive structures from palindromic DNA. D. Leach and J. Lindsey (Edinburgh) (personal communication) report that most of the λ DNA containing a

palindrome is maintained following multiple infection of a *rec*$^+$ *E. coli* strain. Their results, which do not distinguish unreplicated from once-replicated (i.e., semiconserved) duplexes, are compatible with the views presented here as well as with the possibility that palindromes impede replication without sensitizing the DNA to nucleases.

We suggest that the denaturation of palindrome-containing DNA ahead of the replication fork allows the formation of hairpins that cause temporary stalling of DNA polymerase and that are then attacked by *recBC* gene protein and exonuclease I. In the absence of these nucleases the hairpins may be straightened out, for example through the action of the *E. coli* single-strand binding protein, which has been shown to facilitate movement of DNA polymerase III past sites of potential secondary structure *in vitro* (19), permitting replication to continue. The existence of an upper size limit for palindrome stability in a *recBC sbcB* host might then indicate that larger hairpins are not always promptly ironed out, perhaps due to exhaustion of the available supply of single-strand binding protein. This being the case, we could imagine that DNA polymerase crossing the base of a large unresolved hairpin provides the mechanism for symmetrical or near-symmetrical shrinkage of large palindromes. Nonsymmetrical deletion events could occur via this mechanism if DNA synthesis resumed some distance away from the base of the hairpin.

In conclusion, our results confirm earlier work (7) by showing that large palindromes (>2 × 350 bp) are unstable in phage λ growing on even the "permissive", *recBC sbcB*, host. Attempts to maintain a palindrome (2 × 1.6 kilobases) in the prophage state failed, suggesting that neither are large intrachromosomal palindromes tolerated by the *recBC sbcB* host.

The most straightforward explanation for the observed properties of palindromes in *E. coli* involves the formation of secondary structures from palindrome sequences; the driving of palindrome extrusion by the replication machinery would overcome the kinetic barriers believed to otherwise prevent extrusion. In the permissive host, extruded palindromes impede, but do not prevent, replication in a length-dependent fashion. In the wild-type, *rec*$^+$ host, such structures are a signal for chromosome destruction.

We thank David Leach for the gift of DRL107, Flora Banuett for a transposon-linked allele of *hflB*, and Nancy Kleckner for plasmid pNK862, which donates a transposase-deficient kanamycin resistance element to infecting phages. John Clark and David Hagen kindly provided other strains. David Leach proposed the strategy that, with modification, allowed us to propagate a large λ palindrome in the prophage state. We also thank all our colleagues for helpful advice and encouragement and Elizabeth Cooksey for typing the manuscript. The project was supported in part by a Science and Engineering Research Council postdoctoral fellowship (C.E.S.), and by Grant DMB 840943 from the National Science Foundation, and Grant GM33677-01 from the National Institutes of Health (F.W.S.). F.W.S. is an American Cancer Society Research Professor of Molecular Genetics.

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