

Are single-stranded circles intermediates in plasmid DNA replication?

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Plasmid pC194 exists as circular double-stranded and single-stranded DNA in *Bacillus subtilis* and *Staphylococcus aureus*. We report here that the plasmid pHV33, composed of pBR322 and pC194, exists as double- and single-stranded DNA in *Escherichia coli*, provided that the replication functions of pC194 are intact. Single-stranded pHV33 DNA is converted to double-stranded DNA by complementary strand synthesis probably initiated at *rrlB*, a primosome assembly site present on pBR322. The efficiency of complementary strand synthesis affects the double-stranded copy number, which suggests that single-stranded DNA is a plasmid replication intermediate. **Key words: *Escherichia coli*/plasmid pC194/primosome site/single-stranded DNA/single-stranded DNA binding protein**

Introduction

A number of plasmids were recently found to exist as double-stranded and single-stranded DNA in *Bacillus subtilis* and *Staphylococcus aureus* (te Riele *et al.*, 1986). Some of these plasmids are known to replicate also in *Escherichia coli* (Goze and Ehrlich, 1980). We used hybrids between one such plasmid, pC194, and pBR322 to examine single-stranded DNA synthesis in *E. coli*. Single-stranded DNA circles were observed when pC194 replication functions were active. They were converted to double-stranded DNA by complementary strand synthesis initiated at a primosome-assembly site (Arai and Kornberg, 1981). These results suggest that single-stranded circles are intermediates in the replication of pC194.

Results

Single-stranded plasmid DNA in *E. coli*

Single-stranded circular plasmid DNA can be detected in cell lysates as a species which migrates faster through an agarose gel than supercoiled double-stranded monomeric circles and binds to nitrocellulose filters without denaturation of electrophoresed DNA (te Riele *et al.*, 1986). Plasmid pBR322 did not generate single-stranded DNA in *E. coli* by these criteria (Figure 1). On the contrary, plasmid pHV33, composed of pBR322 and pC194 (Figure 2, Primrose and Ehrlich, 1981), generated such DNA (Figure 1). Similarly, plasmid pHV50, consisting of pC194 and the ampicillin resistance gene of pBR322 (Goze and Ehrlich, 1980), produced single-stranded DNA (Figure 1). Its amount was rather high (Figure 1B) considering the low copy number of pHV50 (Figure 1A). These results indicate that pC194 generates single-stranded DNA in *E. coli*.

Characterization of single-stranded plasmid DNA

Single-stranded pHV33 DNA was extracted from *E. coli*, purified by hydroxyapatite chromatography and visualized by electron

microscopy. Circular single-stranded molecules were detected (Figure 3). Thirty molecules were measured and found to be 7.3 ± 0.5 kb long (by comparison with single-stranded f1-pC194 hybrid phage DNA), which corresponds well to the known size

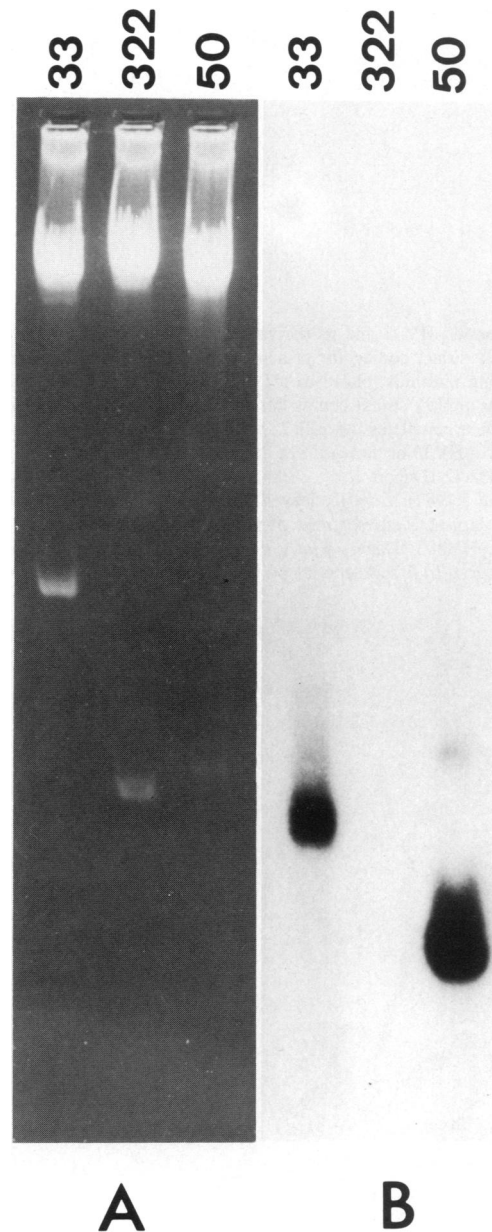


Fig. 1. Detection of single-stranded DNA in *E. coli*. (A) Agarose gel electrophoresis of lysates prepared from *E. coli* containing pHV33, pBR322 and pHV50, respectively. Fast migrating bands correspond to supercoiled double-stranded plasmid monomers. (B) The DNA was transferred to nitrocellulose without prior denaturation and hybridized to ^{32}P -labeled pBR322.

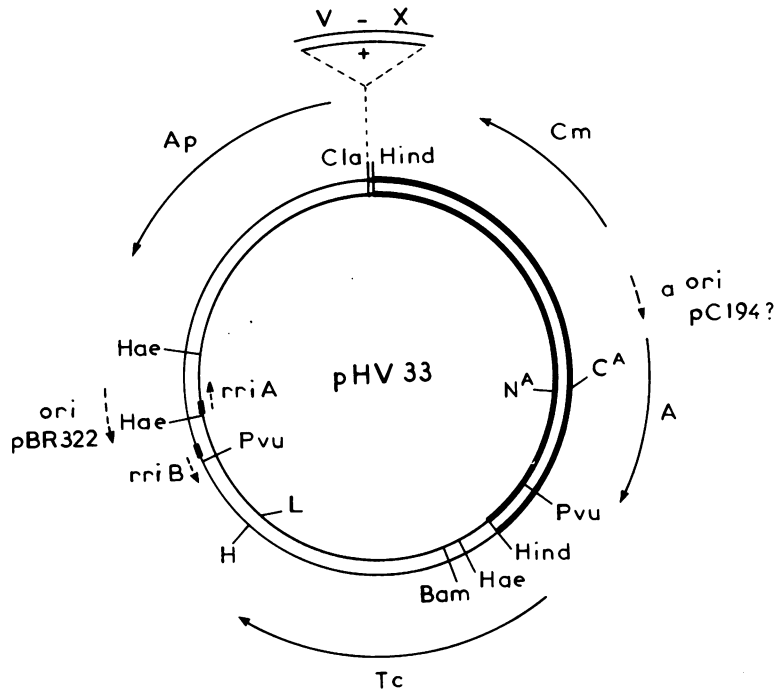


Fig. 2. Plasmid pHV33 and its derivatives. pHV33 consists of pBR322 and pC194 linked at their unique *Hind*III sites such that the pBR322 H-strand is linked to the pC194 strand coding for protein A (C^A). The two zones necessary for pC194 replication are labeled A (an open reading frame) and a (a sequence). The following plasmids related to pHV33 were used: pHV15 (Ehrlich, 1978): pBR322 and pC194 linked at their *Hind*III sites but in the opposite orientation such that the pC194 strand coding for protein A is linked to the pBR322 L-strand. pHV33ΔHae: The largest *Hae*II segment of pHV33 and the adjacent *Hae*II segment which completes the pBR322 replication region. pHV33ΔPvu: The largest *Pvu*II segment of pHV33. pHV33-gene V: 791 bp M13 *Taq*I fragment inserted into pHV33 at the *Cl*aI site located within the pBR322 region. pHV33ΔHae-gene V: 791 bp M13 *Taq*I fragment inserted in the *Cl*aI site of pHV33 Hae. pHV33Δ42 (Dagert *et al.*, 1984): *Bam*HI cleaved pHV33 eroded by BAL31, such that the sequence coding for the protein A is not affected. pHV33Δ81 (Dagert *et al.*, 1984): *Bam*HI cleaved pHV33 eroded by BAL31, such that the sequence coding for the protein A is affected. pHV50 (Goze and Ehrlich, 1980): The largest *Hae*II segment of pHV33. pHV801 (Dagert *et al.*, 1984): pHV33 from which the largest *Hha*I segment is deleted such that the sequence a is missing. pHV803 (Dagert *et al.*, 1984); pHV801 in which sequence a present on a *Hind*III segment of pLJ19 was inserted. pHV805 (Dagert *et al.*, 1984): The two largest *Taq*I segments of pC194 inserted in the *Cl*aI site of pBR322 such that the coding sequence for the protein A is interrupted.

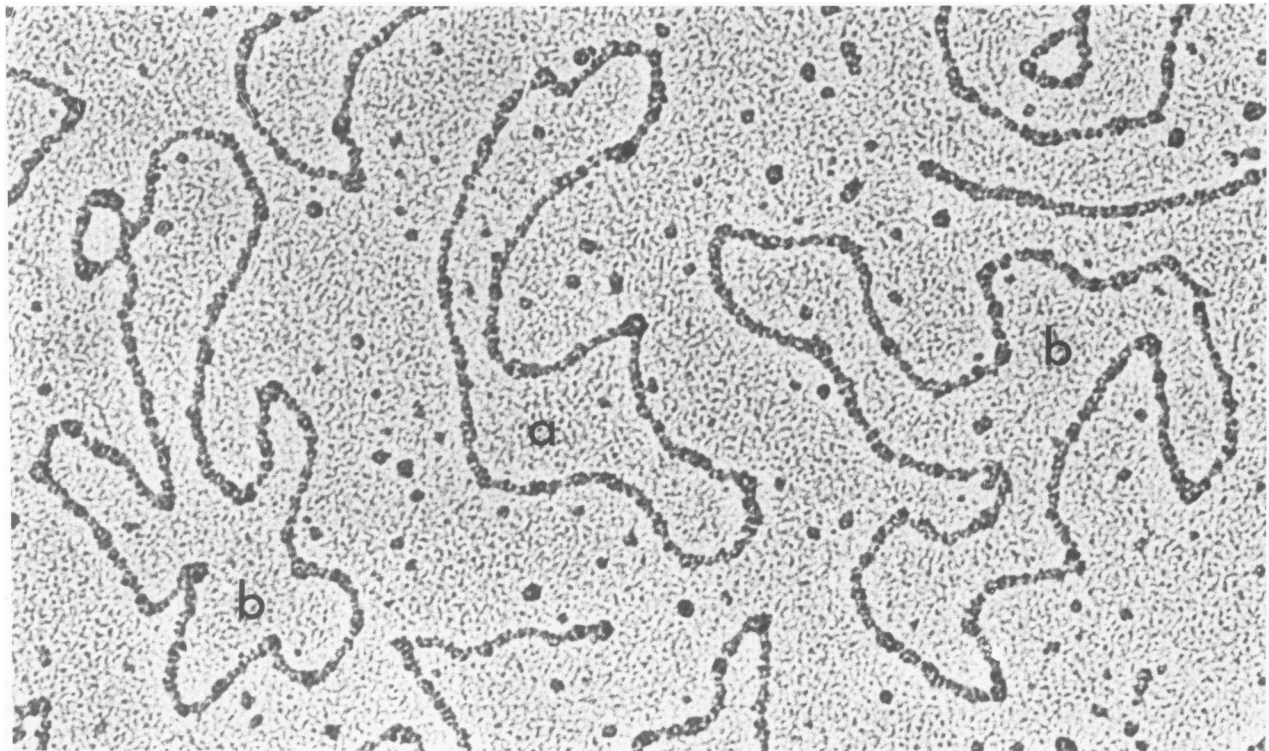


Fig. 3. Single-stranded pHV33 DNA. Single-stranded DNA was extracted from *E. coli* purified by hydroxyapatite chromatography and visualized by electron microscopy. (a) pHV33 DNA; (b) f1-pC194 hybrid phage DNA.

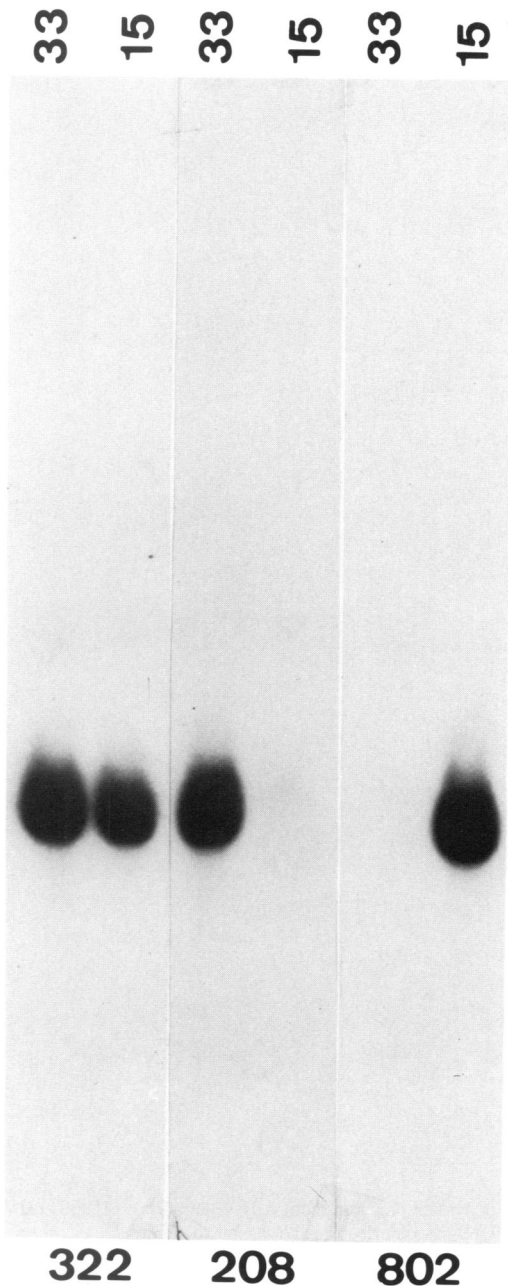


Fig. 4. Hybridization with strand-specific probes. DNA prepared from pHV33- and pHV15-containing *E. coli* was electrophoresed through an agarose gel and transferred to nitrocellulose. Filters were hybridized to ^{32}P -labeled pBR322 DNA, ^{32}P -end labeled single-stranded phage R208 DNA and to ^{32}P -end labeled single-stranded phage R802 DNA.

of pHV33 (7269 bp, determined by sequence analysis of pBR322 and pC194, Sutcliffe, 1978; Horinouchi and Weisblum, 1982; Dagert *et al.*, 1984).

Only one of the two pC194 strands appears as monomeric circles in *B. subtilis* (te Riele *et al.*, 1986). To determine whether the same holds true in *E. coli* two plasmids were used, pHV33 and pHV15, in which pC194 and pBR322 are linked in opposite orientations (Figure 2). Single-stranded DNA from these plasmids, purified by gel electrophoresis, was transferred to nitrocellulose and hybridized to radioactive probes containing the pBR322 L-strand (end-labeled R208 DNA), the H-strand (end-labeled R802 DNA) or both strands (nick-translated pBR322

DNA). The first probe (R208) hybridized with single-stranded pHV33 DNA, the second (R802) with single-stranded pHV15 DNA while the third (pBR322) hybridized with both DNAs (Figure 4), which shows that only the pC194 strand that codes for the protein A (CA, Figure 2) is rendered single-stranded. This result corresponds to that obtained with *B. subtilis* (te Riele *et al.*, 1986), which indicates that in both *B. subtilis* and *E. coli* single-stranded plasmid DNA is generated in the same way.

The presence of single-stranded plasmid DNA depends on pC194 replication functions

The replication region of pC194 consists of two zones, an open reading frame A and a sequence a (Dagert *et al.*, 1984; Figure 2). Inactivation of one of the two, by insertion or deletion, abolishes replication in *B. subtilis*. pHV33 derivatives which contain both zones intact, or in which one of the two zones is inactivated, are described in the legend to Figure 2. These plasmids replicated in *E. coli* at a roughly similar double-stranded copy number (Figure 5A). They generated single-stranded plasmid DNA if the replication region of pC194 was intact but not if either the open reading frame A (pHV33 Δ 81, pHV805) or the sequence a (pHV801) was inactivated (Figure 5B). This shows that the generation of single-stranded DNA depends on pC194 replication functions.

Complementary strand synthesis on the single-stranded plasmid DNA template

pBR322 contains near its replication origin two primosome-assembly sites, named *rriA* and *rriB* (Marians *et al.*, 1982; Nomura *et al.*, 1982), carried on the L-strand and the H-strand of the plasmid, respectively (Figure 2). Complementary strand synthesis on a single-stranded DNA template can be initiated at these sites (van der Ende *et al.*, 1983a).

During pC194-directed replication of pHV33 the H-strand of pBR322 is rendered single-stranded (see above). To determine whether the presence of *rriB* on this strand affects the amount of single-stranded plasmid DNA, two pHV33 deletion derivatives were analyzed. pHV33 Δ Pvu has kept the *rriB* site, while pHV33 Δ Hae has lost it (Figure 2). Both plasmids replicated efficiently in *E. coli* (Figure 6A). Deletion of the *rriB* site resulted in a large increase in the amount of single-stranded DNA (Figure 6B). This result suggests that single-stranded plasmid DNA, generated by replicating activity of pC194, can be converted to double-stranded DNA by complementary strand synthesis initiated by primosome assembly at *rriB*. No single-stranded DNA was observed if pC194 was deleted from pHV33 Δ Hae (not shown).

An even more dramatic increase in the amount of single-stranded plasmid DNA was obtained in the following way: filamentous single-stranded DNA phages produce a single-stranded DNA binding protein, encoded by gene V. A 791-bp *TaqI* fragment of M13, containing gene V and gene X, preceded by a strong promoter (Cashman *et al.*, 1980; Smits *et al.*, 1980), was inserted into pHV33 (pHV33-gene V, Figure 2). This resulted in an increase in the amount of single-stranded plasmid DNA as shown in Figure 6B. If, in addition, the *rriB* sequence was deleted, the amount of single-stranded plasmid DNA was high enough to be easily detected on the ethidium bromide-stained agarose gel (pHV33 Δ Hae-gene V, Figure 6A).

Replication of pHV33 in E. coli polA^s

To investigate the role of complementary strand synthesis in pC194-directed replication, we made use of an *E. coli polA^s* strain, in which the replication region of pBR322 does not function at the restrictive temperature while that of pC194 does (Goze

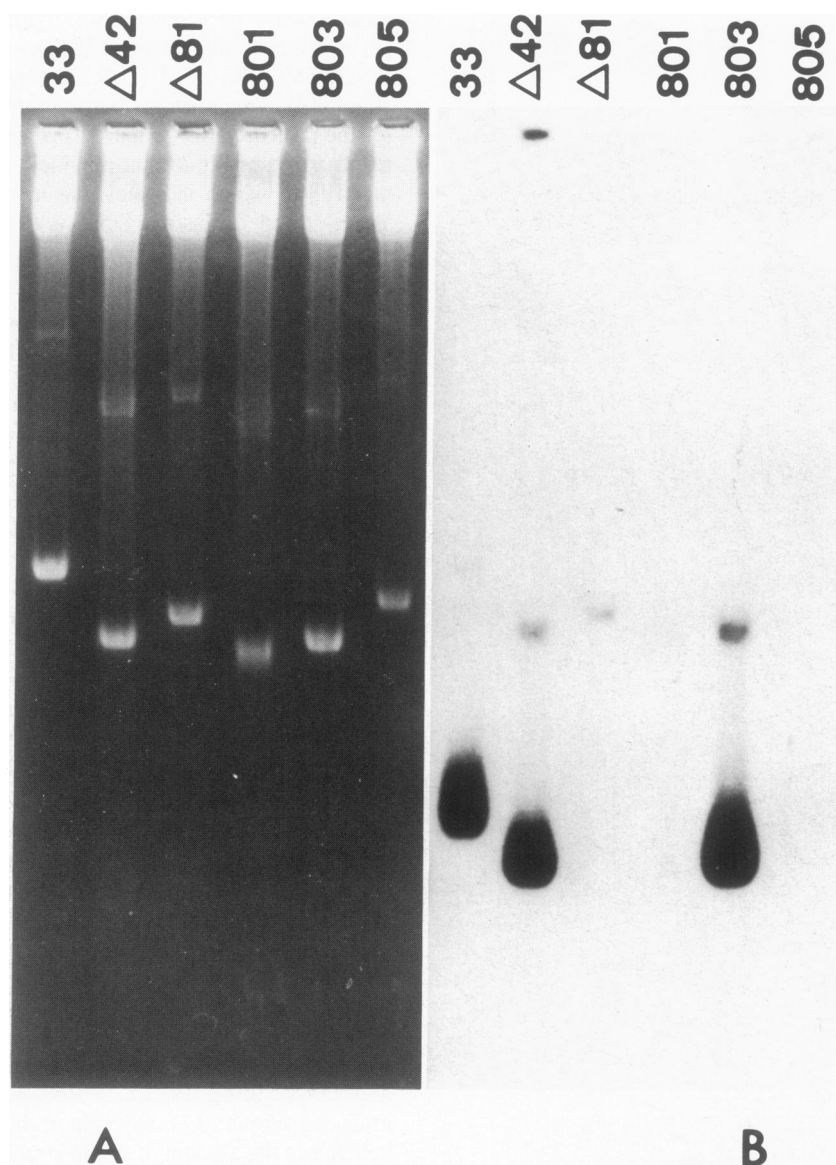


Fig. 5. Analysis of pC194 replication deficient derivatives. (A) Agarose gel electrophoresis of lysates prepared from *E. coli* harboring pHV33, pHV33Δ42, pHV33Δ81, pHV801, pHV803 and pHV805, respectively. (B) Detection of single-stranded DNA as described in the legend to Figure 1.

and Ehrlich, 1980). The *polA*ts strain harboring the plasmids pBR322, pHV33ΔPvu (containing *rriB*) or pHV33ΔHae (lacking *rriB*) was grown at the non-permissive temperature. Lysates were prepared and analyzed. pBR322 did not replicate under these conditions (Figure 7A). Comparison of Figure 7A and 7B shows that in the absence of *rriB*, less double-stranded copies but more single-stranded copies were detected than in the presence of *rriB*. This result suggests that double-stranded copies were generated from single-stranded copies.

Discussion

Generation of single-stranded plasmid DNA

The *S. aureus* plasmid pC194, which replicates in *B. subtilis*, was found to be present as circular single-stranded DNA in both hosts (te Riele *et al.*, 1986). The observation of Goze and Ehrlich (1980) that pC194 also replicates in the Gram-negative bacterium *E. coli* led us to investigate whether or not this plasmid generates single-stranded DNA in this host.

The hybrid plasmid pHV33, an *in vitro* constructed cointegrate of pBR322 and pC194, generated single-stranded DNA in *E. coli*. Single-stranded plasmid DNA was also found in the absence of the replication region of pBR322 (Figure 1) or in a *polA*ts strain at the restrictive temperature (Figure 7), but not in the absence of the replication functions of pC194 (Figure 5). These results show that single-stranded DNA is generated by pC194-directed replication activity in *E. coli*. Since single-stranded plasmid DNA found in *E. coli* had the same characteristics as that found in *B. subtilis* (in both species it was circular and contained the same pC194 strand), it is likely that the above conclusion applies to *B. subtilis* (and probably *S. aureus*) as well. Our results indicate that from the molecules containing pBR322 and pC194 replication regions single-stranded DNA may be generated when one, but not the other, replication region is used. The way in which replication is initiated may therefore determine the form of the DNA molecules synthesized.

The generation of single-stranded plasmid DNA may be indica-

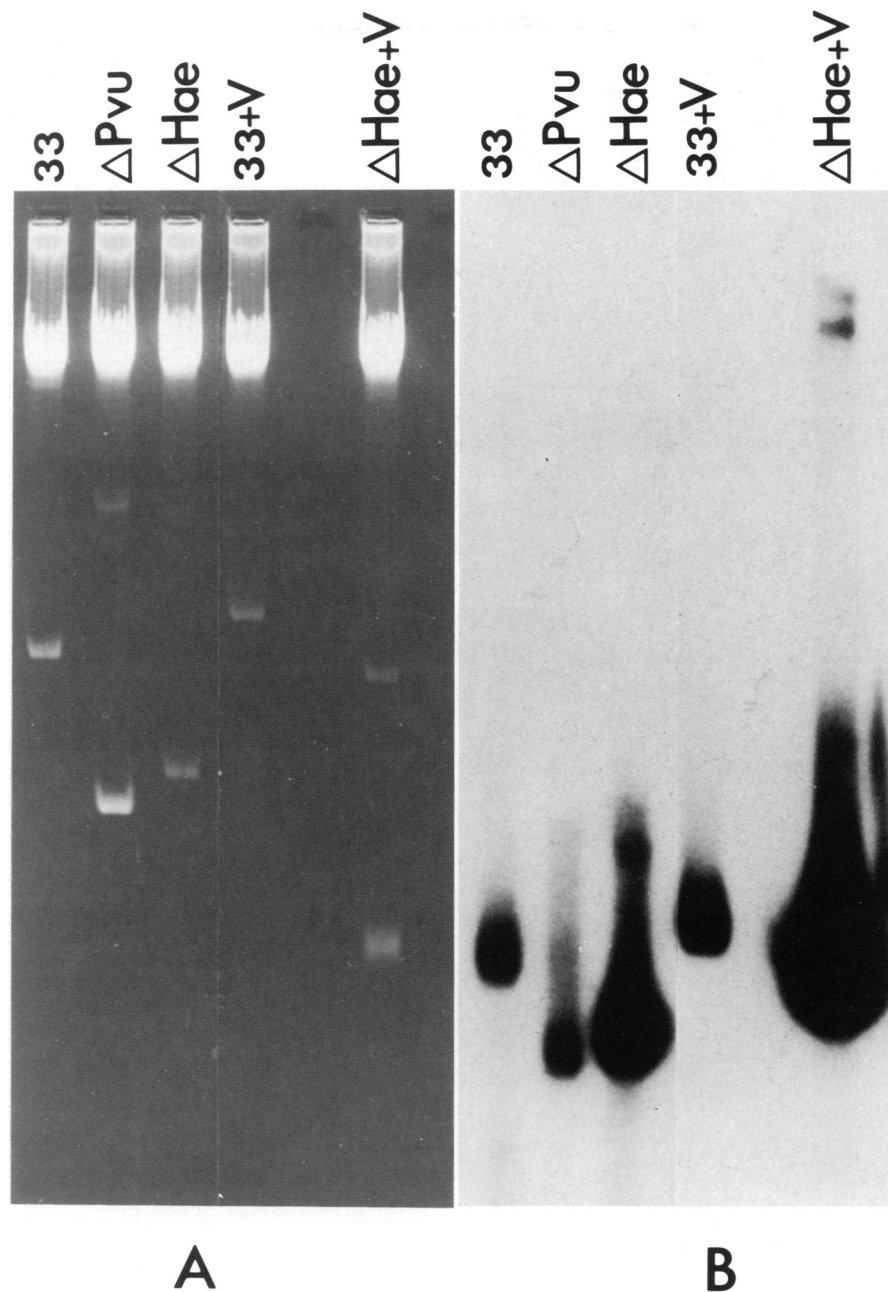


Fig. 6. Primosome assembly site and single-stranded DNA binding protein affect the amount of single-stranded DNA. (A) Agarose gel electrophoresis of lysates prepared from *E. coli* harboring pHV33, pHV33 Δ Pvu, pHV33 Δ Hae, pHV33-gene V and pHV33 Δ Hae-gene V, respectively. (B) Detection of single-stranded DNA as described in the legend to Figure 1.

tive of an asymmetric replication mechanism, in which leading and lagging strand synthesis is uncoupled: a complete single-stranded DNA copy may be displaced if lagging strand synthesis is not initiated before the termination of leading strand synthesis. Displacement synthesis could occur either by the elongation of a 3'-OH end formed by a nick introduced at the origin of replication [similar to the rolling circle mechanism of replication described for single-stranded DNA phages (Schaller, 1978; Horiuchi *et al.*, 1978; Koths and Dressler, 1978)] or by elongation of an RNA primer synthesized at the replication region, resulting in a single-stranded displacement loop [analogous to ColE1-like replication mechanisms (Veltkamp and Stuitje, 1981)]. Our results do not discriminate between the two mechanisms. Recent obser-

vations that *in vitro* replication of *S. aureus* plasmid pT181, which generates single-stranded DNA *in vivo* (te Riele *et al.*, 1986), did not require RNA synthesis (Koepsel *et al.*, 1985a), but that a nick is introduced at the origin of replication by the replication initiation protein (Koepsel *et al.*, 1985b), is suggestive of a rolling-circle-like mechanism. Scheer-Abramowitz *et al.* (1981) claimed, however, the purification and visualization of Θ -shaped replication intermediates formed by another single-stranded DNA producing *S. aureus* plasmid, pE194 (te Riele *et al.*, 1986). Also, the observation of Staudenbauer *et al.* (1979) that uncoupling of leading strand from lagging strand synthesis during ColE1 replication *in vitro* generated single-stranded DNA shows that a D-loop mechanism should not be excluded at present.

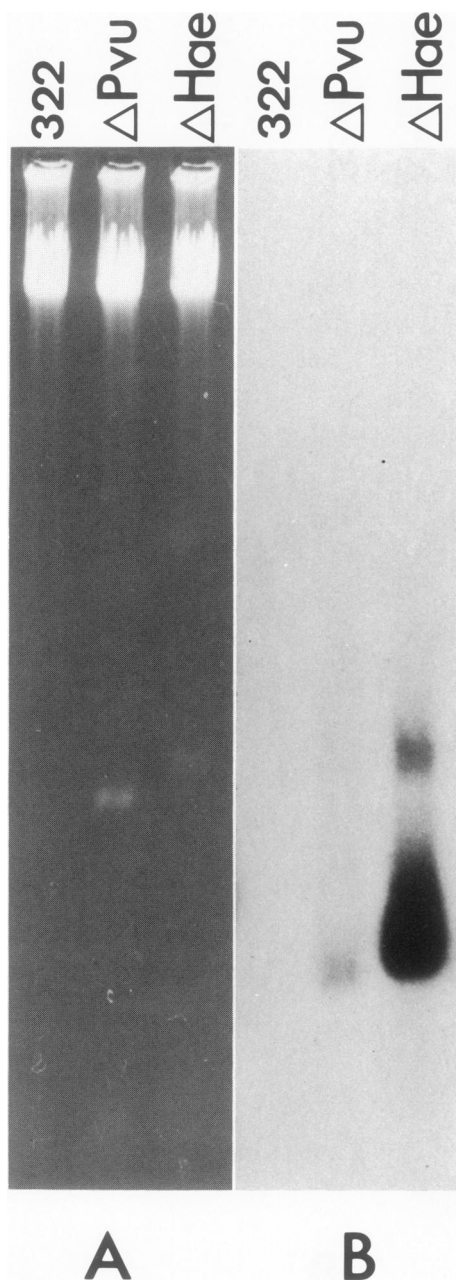


Fig. 7. Single-stranded plasmid DNA generated in *E. coli* cells defective in polymerase I. (A) Agarose gel electrophoresis of lysates prepared from *E. coli* *polA*ts cells harboring pBR322, pHV33ΔPvu and pHV33ΔHae, respectively, grown at the non-permissive temperature (42°C). (B) Detection of single-stranded DNA as described in the legend to Figure 1.

Complementary strand synthesis on single-stranded DNA

Single-stranded bacteriophage DNA is converted to double-stranded DNA by complementary-strand DNA synthesis, initiated by the generation of a short RNA primer. Bacteriophage G4 requires the host *dnaG* gene product (Wickner, 1978) whereas the filamentous phages use RNA polymerase (Gray *et al.*, 1978; Geider *et al.*, 1978) for priming at a specific site in the origin of replication. A much more complex system is used by ΦX174, where primer formation requires the assembly of a multi-enzyme complex, called the primosome, at a specific site on the single-stranded DNA template. The primosome can move along the

DNA template and generate RNA primers at non-specific sites (McMacken *et al.*, 1977; Arai and Kornberg, 1981). Initiation signals for complementary strand DNA synthesis have been detected in a number of replicons, including the *E. coli* chromosome (van der Ende *et al.*, 1983a). Plasmid pBR322 contains on each strand a primosome assembly site [called Y effector site or n' protein activator site (Zipursky and Marians, 1980, 1981; Shlomaï and Kornberg, 1980)], designated *rriA* in the L-strand and *rriB* in the H-strand (Marians *et al.*, 1982; Nomura *et al.*, 1982). Although neither of these sites is required for the replication of pBR322 (van der Ende, 1983b), complementary strand synthesis on a single-stranded DNA template can be initiated at both (van der Ende *et al.*, 1983a). The observation that deletion of the H-strand site results in a dramatic increase in the amount of single-stranded plasmid DNA (Figure 6) suggests that single-stranded pHV33 DNA can be converted to a double-stranded form *via* a primosome-dependent initiation mechanism. Overproduction of a single-stranded DNA-binding protein in the cell, by cloning the M13 gene V on hybrid plasmids, led to an even larger increase in the amount of single-stranded DNA, up to 70% of the total plasmid DNA (Figure 6). This effect may be due to interference with the conversion of single- to double-stranded DNA or to protection of single-stranded DNA against the host nucleases.

An inverse correlation between the amount of double- and single-stranded DNA of hybrid plasmids composed of pC194 and pBR322 was observed (Figure 6). The presence of two replication regions on these genomes makes it difficult to interpret this result. We therefore made use of a *polA*ts strain in which pBR322 replication is blocked at the restrictive temperature while pC194 replication functions are not affected. Deletion of *rriB* decreased the amount of double-stranded DNA copies and increased the amount of single-stranded copies (Figure 7). This result supports the hypothesis that pC194 replicates *via* a single-stranded intermediate which accumulates in the absence of efficient complementary strand synthesis. In *E. coli* the presence of the *rriB* site provides efficient primosome-dependent priming of complementary strand synthesis. Other, probably less efficient, initiation mechanisms might be used as well (Arai *et al.*, 1981), which accounts for the detection of double-stranded DNA in the cells harboring plasmids devoid of *rriB*, such as pHV50 (Figure 2) or pHV33ΔHae, under *polA*⁻ conditions (Figure 7).

Materials and methods

Bacteria, plasmids and phages

E. coli strains HVC45 (R.Davies) and HVC1 (*polA*214ts, D.Helsinki) were used as hosts for plasmids pBR322 (Bolivar *et al.*, 1977), pHV33 (Primrose and Ehrlich, 1981) and its derivatives which are described in the legend to Figure 2. Cells were grown in LB containing 100 µg/ml of ampicillin at 37°C or 42°C.

Bacteriophages R208 (Boeke *et al.*, 1979) and R802 (te Riele *et al.*, 1986) consist of f1 DNA and pBR322 DNA. Single-stranded R208 DNA, extracted from phage particles, contains the pBR322 L-strand; single-stranded R802 DNA contains the H-strand.

Preparation of cell lysates and gel electrophoresis

Growing cells were harvested at OD 650 ~ 1, washed with Tris 50 mM, EDTA 5 mM, NaCl 100 mM, pH 8.0, resuspended in lysis buffer containing Tris 40 mM, EDTA 2 mM, Na-acetate 20 mM, pH 8.0, plus SDS 1% and Ficoll 2% and heated for 30 min at 65°C as described by Twigg and Sheratt (1980).

The lysates were extracted with phenol, treated with RNase I and electrophoresed through agarose gels as described (te Riele *et al.*, 1986).

Detection and analysis of single-stranded plasmid DNA

Methods to detect single-stranded plasmid DNA by Southern hybridization, end-labeling of single-stranded hybridization probes and nick-translation, purification and visualization of single-stranded DNA by electron microscopy have been described (te Riele *et al.*, 1986).

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