The EMBO Journal vol.6 no.12 pp.3863–3869, 1987

Rolling circle replication of single-stranded DNA plasmid pC194

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Communicated by S.D.Ehrlich

A group of small *Staphylococcus aureus/Bacillus subtilis* plasmids was recently found to replicate via a circular single-stranded DNA intermediate (te Riele *et al.*, 1986a). We show here that a 55 bp region of one such plasmid, pC194, has origin activity when complemented in *trans* by the plasmid replication protein. This region contains two palindromes, 5 and 14 bp long, and a site nicked by the replication protein. DNA synthesis presumably initiated at the nick in the replication origin can be terminated at an 18 bp sequence homologous to the site of initiation, deriving from another plasmid, pUB110, or synthesized *in vitro*. This result suggests that, similar to the *Escherichia coli* single-stranded DNA phages, pC194 replicates as a rolling circle. Interestingly, there is homology between replication origins and replication proteins of pC194 and the phage ΦmX174.

Key words: replication origin/single-stranded DNA/phage/plasmid homologies

Introduction

The plasmid pC194 belongs to a group of small *Staphylococcus aureus/Bacillus subtilis* plasmids, whose replication depends on a plasmid encoded replication initiator protein (Novick *et al.*, 1982). We have recently demonstrated that these plasmids exist intracellularly as double-stranded and single-stranded DNA (ds DNA, ss DNA) circles (te Riele *et al.*, 1986a). Further analysis of pC194, which also replicates efficiently in *Escherichia coli* (Goze and Ehrlich, 1980; unpublished results), and generates circular ss DNA in this host (te Riele *et al.*, 1986b), showed that ss DNA consists of only one of the DNA strands and that its formation requires the plasmid replication protein. When a signal which stimulates the conversion of ss DNA to ds DNA, is present on the single-stranded template, the amount of ss DNA decreased, whereas the double-stranded copy number increased (te Riele *et al.*, 1986b; Gruss *et al.*, 1987; unpublished results). These observations suggest that pC194-like plasmids replicate asymmetrically: displacement synthesis starting at the plasmid 'plus-origin' generates circular ss DNA, which is then converted to ds DNA by complementary strand synthesis initiated at a 'minus-origin' elsewhere on the single-stranded template.

Replication of the rolling circle type such as that described for ss DNA phages of *E.coli* generates ss DNA (Horiuchi *et al.*, 1978; Koths and Dressler, 1978; Schaller, 1978). In these genomes initiation of replication occurs by the introduction of a nick by the phage-encoded nicking–closing enzyme at the plus-origin of replication. The 3′-OH end thus generated is used as a primer for displacement DNA synthesis. Replication terminates by cleavage of the nascent single-strand from the replication intermediate, and religation of its extremities. Necessarily, initiation and termination occur at the same site in the replication origin. However, when two phage origins are present on the same replicon, initiation can occur at one origin and termination at the other, resolving such a replicon into two smaller molecules with recombined origins (Dotto and Horiuchi, 1981; van der Ende *et al.*, 1982). If one origin contains only an active signal for termination, but not for initiation, only one recombinant molecule is formed (Dotto *et al.*, 1982; Peeters *et al.*, 1987).

One of the ss DNA producing plasmids, pT181 (te Riele *et al.*, 1986a), is known to be nicked at the replication origin by the plasmid replication protein (Koepsel *et al.*, 1985). Evidence has been presented which indicates that also pC194 is nicked at a specific site within the replication region by its replication protein (Michel and Ehrlich, 1986a). On the basis of these results it was suggested that, similar to ss DNA phages the two plasmids replicate via a rolling circle mechanism (te Riele *et al.*, 1986b; Koepsel *et al.*, 1985; Michel and Ehrlich, 1986a; Khan *et al.*, 1982). It follows from that suggestion that their replication terminates at an appropriate signal. We tested that prediction with the plasmid pC194. For that purpose we first mapped the plasmid origin of replication to a 55 bp sequence which contains the putative nick site. We then showed that 18 bp corresponding to the sequence flanking the nick site in the origin may be used as a signal for the termination of replication. This result conforms to the prediction made, and indicates that pC194 replicates as a rolling circle.

Results

Deletion analysis of the pC194 replication origin

To determine the limits of the pC194 replication origin, we used deletion derivatives of the plasmid pHV33. This plasmid is composed of pBR322 and pC194 (Primrose and Ehrlich, 1981; Figure 1A), and replicates in *E.coli* using either the pBR322 or pC194 replication functions and in *B.subtilis* using the pC194 replication functions (pBR322 does not replicate in this host; Goze and Ehrlich, 1980; te Riele *et al.*, 1986b; Ehrlich, 1978). Deletion derivatives were isolated in *E.coli* and tested for replication activity in *B.subtilis*. They were considered to carry a functional pC194 replication origin if they transformed *B.subtilis* and yielded transformants harbouring a plasmid indistinguishable from the transforming one by biological and physical properties (conferring the expected phenotype upon *E.coli* and *B.subtilis*; having the expected size and restriction patterns with several enzymes).

Five pHV33 derivatives contained the complete replication gene A, localized downstream from the nick (downstream and upstream refer to sequences replicating early and late, respectively) and only a short pC194 sequence (1–74 bp) upstream from the nick (Figure 1B). These plasmids were genetically labelled by the erythromycin (Em) resistance gene of the plasmid pE194. The two plasmids in which 74 or 25 bp of the upstream sequence remained (labelled Δ-74 and Δ-25, Figure 1B) could replicate in *B.subtilis*; the plasmids in which 5 bp or less remained (Δ-5, Δ-4, Δ-1, Figure 1B) could not.

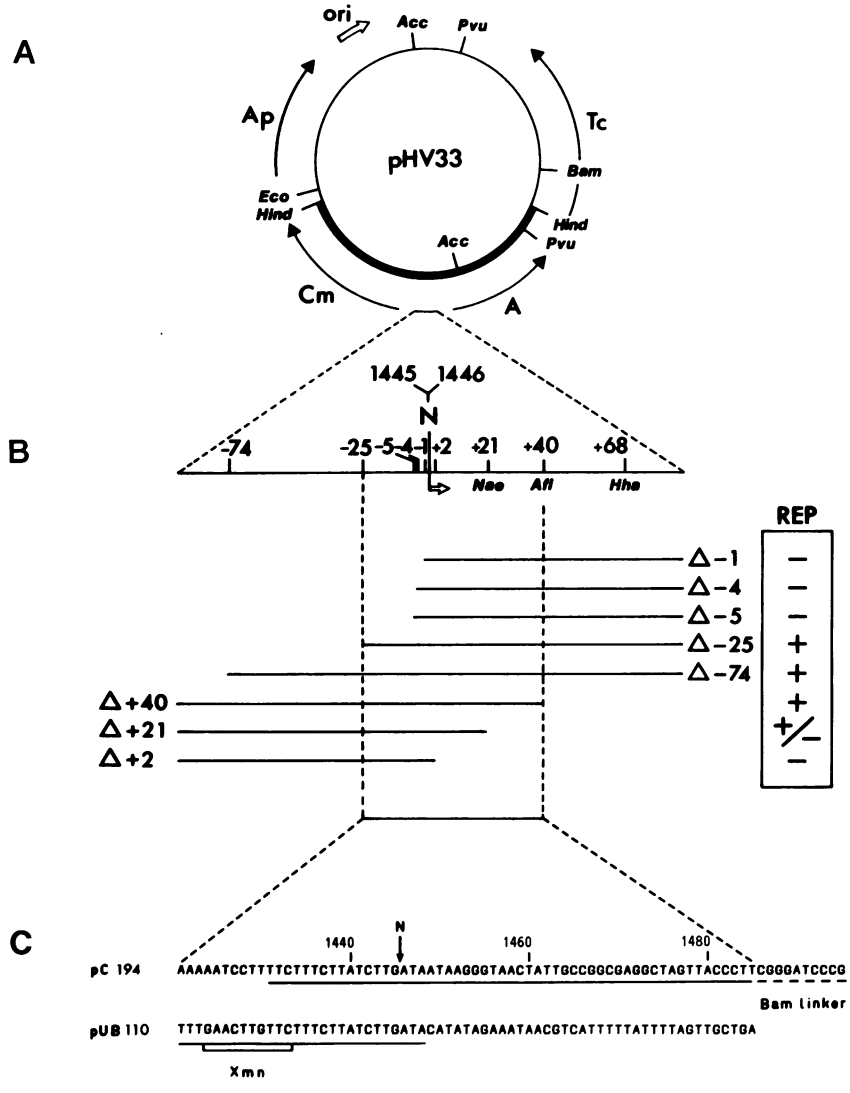

Fig. 1. Plasmid pHV33 and its derivatives. (**A**) pHV33. The thin line represents pBR322, the thick line pC194. Antibiotic resistance genes are indicated by arrows. The open reading frame A encodes the pC194 replication protein. (**B**) Schematic representation of the pC194 origin region between the Cm-resistance gene and the gene A. The nick site (N) between nucleotides 1445 and 1446 of the pC194 map (numbering according to Dagert *et al.*, 1984) and the direction of replication (⇒) are indicated. Numbers refer to nucleotide positions upstream (−) and downstream (+) of the nick site. Lines represent sequences present in pHV33 deletion derivatives. The box REP indicates the origin activity. (**C**) The origin region of pC194 aligned with a homologous pUB110 sequence. The underlined sequence represents the origin, resulting from initiation of DNA replication at the pC194 origin and termination at the homologous part of the pUB110 sequence (see text). The *Bam*HI linker inserted at position +40 relative to the nick is indicated.

Three pHV33 derivatives carried the chloramphenicol (Cm) resistance gene localized upstream from the nick and only a short pC194 sequence (2−40 bp) downstream from the nick (Figure 1B). Since they lacked the gene A, examination of their replication activity required that the corresponding protein be provided in *trans*. We used for that purpose a plasmid, labeled pHV1020, composed of the pC194 segment encoding the gene A (delimited by the *Hha*I and *Pvu*II sites at nucleotides 1513 and 2592, respectively) and the replicon of pUB110. This plasmid has no extensive homology with the three pHV33 deletion derivatives, and could therefore not recombine with them. The two plasmids in which 40 and 21 bp of pC194 sequence downstream from the nick remained (Δ+40, Δ+21, Figure 1B) replicated in *B.subtilis* cells harboring pHV1020, while the plasmid in which only 2 bp remained (pHV60, Figure 1B) did not. None of the plasmids replicated in *B.subtilis* harbouring only the vector plasmid pUB110, which indicates that they required the product of gene A. The copy number of the two replicative plasmids which carried the gene A (Δ-74, Δ-25) was comparable to that of pHV33 (Figure 2).

The copy number of the plasmid Δ+40, complemented in *trans*, was higher (Figure 2), possibly because the protein A was overproduced by the complementing plasmid pHV1020. On the contrary, the copy number of the plasmid Δ+21 which was also complemented in *trans* was low. These results indicate that the pC194 replication origin is contained within a 65 bp region (−25 to +40) and that a shorter region (−25 to +21) may function but with a lower efficiency.

Cloning of the replication origin

Inspection of the nucleotide sequence of the pC194 origin revealed that the nick is introduced within an 18 bp sequence also present in plasmid pUB110 (Figure 1C; Horinouchi and Weisblum, 1982b; Hahn and Dubnau, 1985). Efficient intramolecular recom-

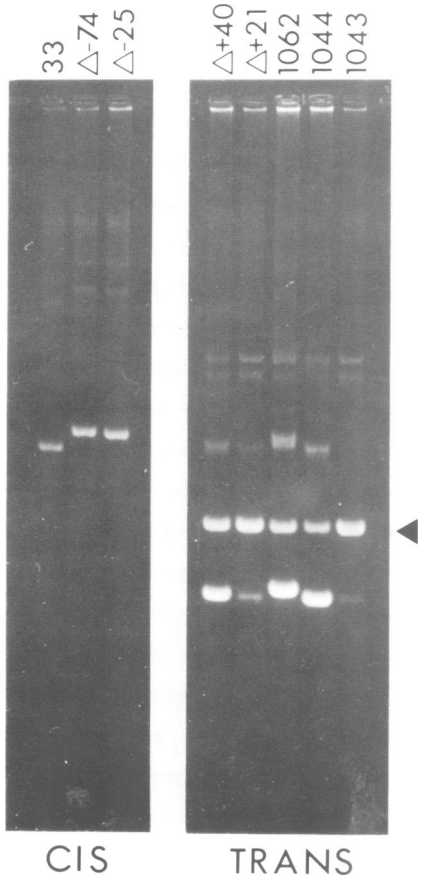

Fig. 2. Replication activity of pHV33 deletion derivatives in *B.subtilis*. Competent *B.subtilis* cells were transformed with the pHV33 deletion derivatives shown in Figure 1B. DNA was extracted and analyzed by agarose gel electrophoresis. *Cis* and *trans* refer to plasmids containing the gene A and plasmids replicating in the presence of the A protein donor pHV1020 (indicated by a triangle), respectively.

bination (which, as we demonstrate below, corresponds to the termination of plasmid DNA replication) between the pC194- and pUB110-carried 18 bp sequence allowed us to construct *in vivo* a plasmid carrying a putative pC194 origin. This origin, corresponding to a sequence between position −15 and +40 relative to the nick is flanked by an *Xmn*I site (deriving from pUB110) and a *Bam*HI linker sequence inserted at position +40 relative to the nick of pC194 (Figure 1C, plasmid labelled pHV1061). The *Xmn*I/*Bam*HI segment was cloned in plasmid pUC18 and the resulting plasmid was genetically labelled by the Em-resistance gene of plasmid pE194 (pHV1062, see Table I). This plasmid replicated efficiently in *B.subtilis* cells harbouring a helper plasmid providing the gene A protein (pHV1020 was used) as judged by its high copy number (Figure 2). It failed to replicate in cells devoid of a helper plasmid (harbouring pUB110). This indicates that the pC194 replication origin may correspond to the cloned 55 bp plasmid region.

Secondary structure of the replication origin

Two palindromes of 5 and 14 bp are present within the 55 bp pC194 replication origin. They may be forming the hairpin structures shown in Figure 3C, as suggested by the following evidence.

The pC194 sequence downstream from nucleotide +40 relative to the nick is not needed for origin function since the plasmid which contained a 10 bp *Bam*HI linker inserted at that site (pHV1042) or the plasmids in which the downstream pC194 se-

Table I. List of plasmids[a]

Plasmid	Structure or construction[a]	Reference
pBR322	Cloning vector for *E.coli*	Bolivar *et al.* 1977
pC194	Natural isolate from *S.aureus*	Iordanescu, 1975
pE194	Natural isolate from *S.aureus*	Iordanescu, 1977
pUB110	Natural isolate from *S.aureus*	Gryczan *et al.* 1978
pUC18	Cloning vector derived from pBR322	Yanish-Perron *et al.*, 1985
pHV15	pBR322 and pC194 linked at their *Hin*dIII sites	Ehrlich, 1978
pHV33	pBR322 and pC194 linked at their *Hin*dIII sites, but in opposite orientation to pHV15	Primrose and Ehrlich 1981
pHV33 Δ-74 Δ-25 Δ-5 Δ-4 Δ-1	Deletions of the Cm-resistance gene of pHV33, ending at different positions upstream of the pC194 nick site. The plasmids were marked by the pE194 Em-resistance gene	Michel and Ehrlich, 1986a, b, this work
pHV33 Δ+40 Δ+21	*In vitro* constructed deletions of the open reading frame A, ending at different positions downstream from the pC194 nick site	This work
pHV60	*In vivo* deletion in pHV33 of the open reading frame A, ending 2 nucleotides downstream from the pC194 nick site	Michel and Ehrlich 1986a, Primrose and Ehrlich, 1981
pHV1015	Insertion of a *Bam*HI linker at the *Hha*I site of pC194	This work
pHV1020	pC194 gene A inserted into pUB110	"
pHV1025	pE194 Em-resistance gene inserted in pBR322	"
pHV1041	Insertion of TTA at the *Afl*II site in pHV33	"
pHV1042	Insertion of a *Bam*HI linker at the *Afl*II site in pHV33	"
pHV1043	The largest *Acc*I fragment of pHV1041	"
pHV1044	The largest *Acc*I fragment of pHV33	"
pHV1045	Insertion of a *Bam*HI linker at the *Afl*II site in pHV15	"
pHV1050	900 bp *Sau*3A segment C of pUB110 inserted in the *Bam*HI site of pHV15	"
pHV1051	As pHV1050, but with a *Bam*HI linker inserted at the pC194 *Afl*II site	"
pHV1052	As pHV1050, but deletion of a 400 bp *Nsi*I segment inactivating the gene A	
pHV1053	900 bp *Sau*3A segment C of pUB110 inserted in the *Bam*HI site of pHV33	"
pHV1055	Synthetic 18 bp sequence inserted between the *Sal*I and *Eco*RV sites of pHV15	"
pHV1057	Synthetic 18 bp sequence inserted between the *Sal*I and *Nru*I sites of pHV33	"
pHV1061	pHV1051 derivative containing a reconstituted pC194 origin region flanked by an *Xmn*I and a *Bam*HI site	"
pHV1062	Insertion of the pC194 origin region of pHV1061 in pUC18, marked by the pE194 Em^R gene of pHV1025	"

[a]Complementary relevant details are described in the text.

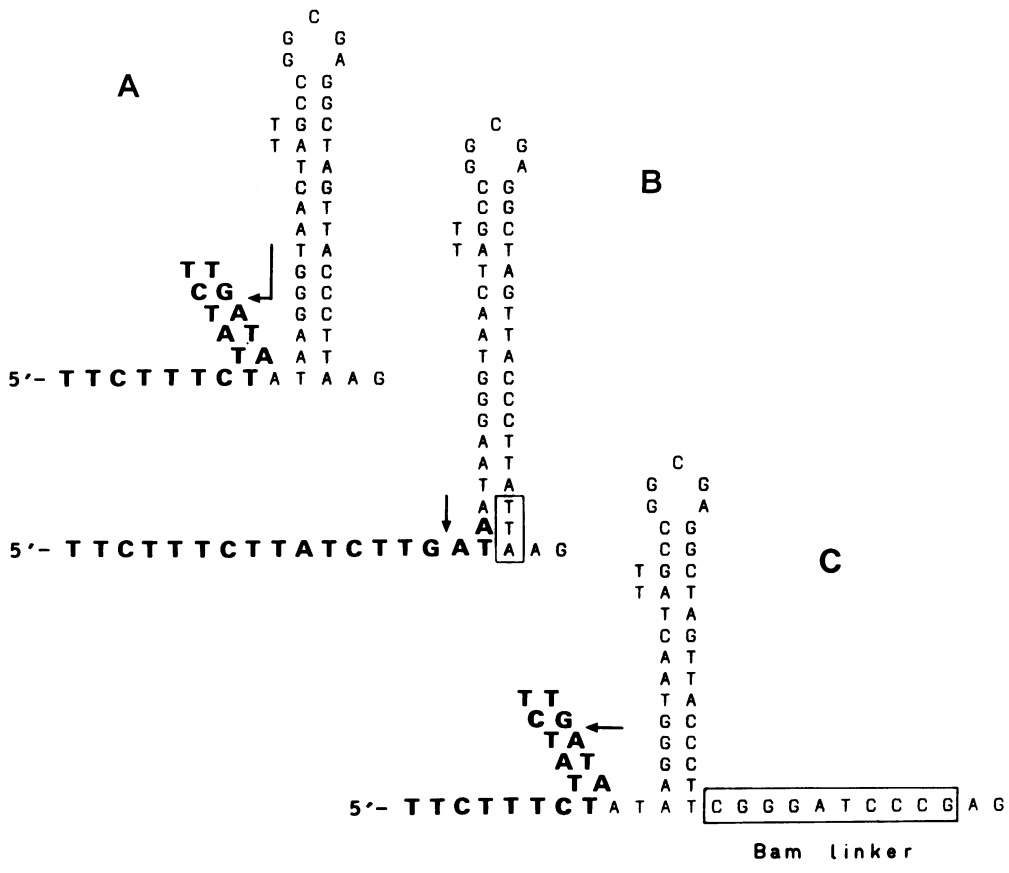

Fig. 3. Hairpin structure of the pC194 origin region in the wild-type pC194 (**A**), the plasmid containing the TTA insertion at position 41 (**B**), the plasmids containing a *Bam*HI linker at position +40 (**C**). The arrow indicates the position of the nick.

quences were replaced by pBR322 sequences (pHV33 +40 and pHV1062) could all replicate in *B.subtilis* (the last two are shown in Figure 2). Yet, insertion of 3 base-pairs, TTA, within this superfluous region, at position +41 interfered with the origin function, since the resulting plasmid, pHV1041, did not transform *B.subtilis*. In this plasmid the larger hairpin could be extended at the expense of the smaller (Figure 3B). Such interaction is not possible in the wild-type plasmid (in which the larger palindrome is 15 bp long, Figure 3A), or in the other three plasmids having the region downstream of position +40 modified (an example is shown in Figure 3C).

The origin mutated by the TTA insertion was not completely inactive, as shown by the following complementation assay. The gene A was inactivated in pHV1041 and pHV33 by a deletion which gave plasmids pHV1043 and pHV1044, respectively. Both plasmids transformed *B.subtilis* containing the plasmid pHV1020 (which provided the protein A), but the copy number of the TTA insertion mutant was very low (Figure 2).

Termination of DNA synthesis

Rolling circle DNA replication, observed with certain ss DNA phages, can terminate precociously at a sequence homologous to the phage replication origin (Dotto *et al.*, 1982; Peeters *et al.*, 1987). Genomes smaller than the parental are generated by this process. To examine whether pC194 replication can terminate in a similar way we propagated in *E.coli* (a host in which pC194 replication is active; Goze and Ehrlich, 1980; te Riele *et al.*, 1986b) plasmids which carried in addition to the pC194 replication origin a sequence homologous to the origin and searched for the presence of smaller derivatives, indicative of rolling circle replication. Plasmid pUB110 carries a sequence homologous to the pC194 replication origin (Figure 1C; Hahn and Dubnau, 1985). We constructed plasmids pHV1050 and pHV1053, composed of pBR322, pC194 and *Sau*3A segment C of pUB110 which contains that sequence (Figure 4A). The homologous sequences were directly repeated in the two plasmids. Both pHV1050 and pHV1053 were always accompanied by a smaller derivative even when they were purified by gel electrophoresis and re-introduced in *E.coli* by transformation (a representative plasmid analysis is shown in Figure 4B). The small plasmids deriving from pHV1050 and pHV1053 were isolated and analysed by restriction and sequencing. Their structures are indicated in Figure 4A. In both cases, they corresponded to that expected from the initiation of DNA synthesis at the nick site within the pC194 replication origin and termination at the strictly homologous 18 bp region of the pUB110 sequence (Figure 1). The smaller plasmids were not generated if the pC194 replication gene A was inactivated by deletion (plasmid pHV1052, Figure 4B), which suggests that they resulted from plasmid replication.

Could the small plasmids accompanying pHV1050 and pHV1053 be generated by recombination between the two homologous 18 bp sequences, rather than by pC194 replication? Several observations argue against that interpretation. First, recombination should generate from each parental plasmid two rather than only one progeny plasmid. The missing progeny plasmid expected from pHV1050 could be difficult to detect if recombination were inefficient, since it would not be replicative (it would carry neither the pC194 nor the pBR322 replication origin, Figure 4A). On the contrary, the missing progeny plasmid expected from pHV1053 should be easy to detect since it would be replicative (it would carry the pBR322 replication region,

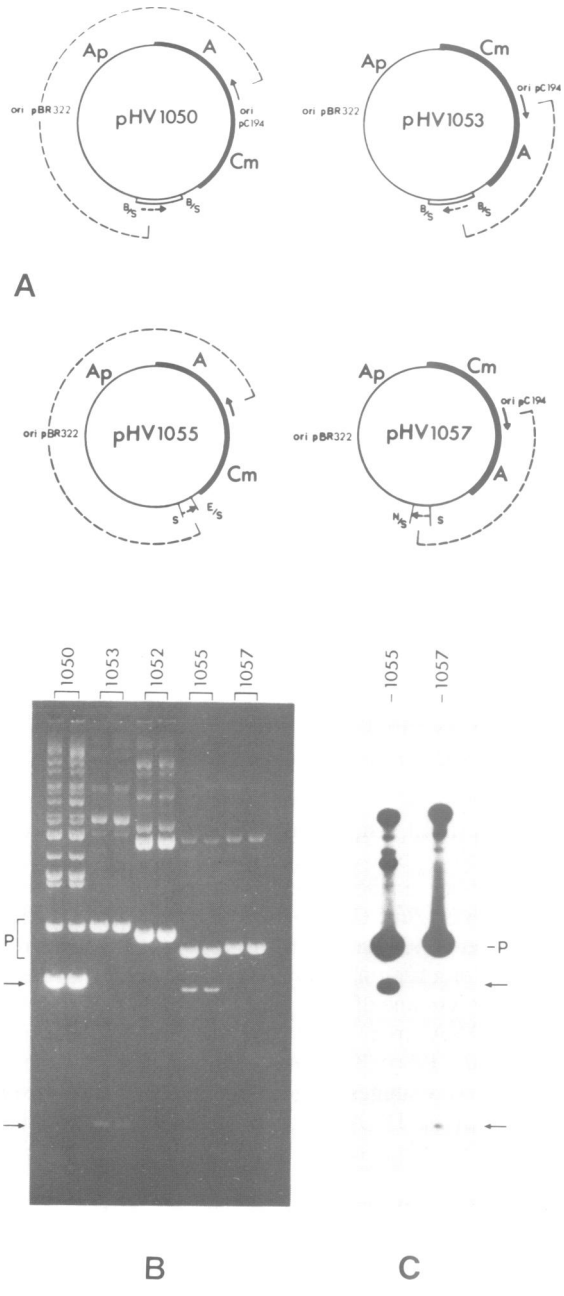

Fig. 4. Termination of pC194 replication. (**A**) Plasmids consisting of pBR322 (thin line), pC194 (thick line) and an additional copy of the 18 bp sequence flanking the nick of pC194 (--→). The orientation of the additional copy, carried within the 900 bp *Sau*3A sequence of pUB110 (pHV1050 and pHV1053) or synthesized *in vitro* (pHV1055 and pHV1057), is the same as that of the origin copy (→). Direction of pC194 replication is indicated by the arrow. B/S, E/S, N/S and S refer to sites used for plasmid construction (cf Materials and methods) (**B**) Purified plasmids monomers were introduced into *E.coli*, and their DNA was analyzed by gel electrophoresis (two extractions for each plasmid are shown). pHV1052 is a rep$^-$ derivative of pHV1050. (**C**) Plasmids pHV1055 and pHV1057 were analyzed by Southern hybridization, using as a probe the pC194 fragment between positions 1513 and 2592, carrying the gene A. P indicates the positions of the parental plasmids, arrows indicate the positions of smaller derivatives.

Figure 4A). Second, recombination between the two homologous sequences present in the opposite rather than the same orientation on a plasmid should invert the segments delimited by these sequences. No inversion was found in two such plasmids (not shown). Finally, RecA protein was not involved in the generation of the small plasmids, since their amount was the same in rec$^+$ and *recA E.coli* cells (not shown).

To test whether the 18 bp sequence common to pC194 and pUB110 can be used as a signal for termination of pC194 DNA synthesis in the absence of the flanking pUB110 region we constructed plasmids pHV1055 and pHV1057 composed of pBR322, pC194 and the 18 bp sequence, synthesized *in vitro* (Figure 4A). These plasmids were always accompanied by smaller derivatives (Figure 4B and C) which had a structure (determined by restriction and sequence analysis) expected for a plasmid generated by intiation at the pC194 replication origin and termination at the synthetic 18 bp sequence. This sequence was therefore recognized as a signal for termination of the pC194 replication in the absence of the flanking pUB110 region.

Discussion

Previous work has indicated that a nick is introduced in the pC194 genome by the plasmid replication protein between nucleotides 1445 and 1446 (Michel and Ehrlich, 1986a). We show here that deletions ending 5 bp upstream or 2 bp downstream from the nick abolish the replication activity of the plasmid, while those ending 25 bp upstream or 40 bp downstream do not. A somewhat shorter sequence, between positions −15 and +40 relative to the nick, had the origin activity when complemented in *trans* by the pC194 replication protein. These results show that the plasmid replication origin is localized in a 55 bp region containing the nick site.

The 55 bp region has the potential to fold into two hairpins, with stems of 5 and 14 bp (15 bp if the region is extended 1 bp, as in the wild-type sequence), and loops of 2 and 5 bp, respectively (Figure 3). Formation of the hairpins may be important for origin activity, since a mutation outside of the 55 bp origin region which allows the extension of the large hairpin at the expense of the small one (insertion TTA, Figure 3B) interfered with the origin activity while other nearby changes, which could not modify the hairpin structure, did not. Furthermore, a deletion eliminating a part of the large palindrome (Δ+21, Figure 1), and thus interfering with the hairpin formation, drastically reduced origin activity. An additional support for the role of the hairpins comes from the studies of illegitimate recombination catalysed by the pC194 replication protein (Michel and Ehrlich, 1986a). Recombination was found to occur at a consensus sequence resembling that of the 5 bp hairpin (Figure 3), in which the loop (TT) and the adjacent base pair (CG in the origin, CG or TA in the consensus sequence) were almost always present.

It is interesting that the 18 bp sequence at the 5′ end of the pC194 replication region is identical to a sequence carried by the plasmid pUB110, and that 13 out of the following 15 pC194 nucleotides are identical to 13 out of the following 20 pUB110 nucleotides (Hahn and Dubnau, 1985; Figure 1). If the conservation of the sequence corresponds to a conservation of function, the pUB110 replication origin is situated at the site homologous with the pC194 replication origin (close to nucleotide 4294; numbering according to McKenzie *et al.*, 1986). The fact that the replication proteins of pC194 and of pUB110, which must interact with the plasmid replication origins, are fairly homologous (close to 30%, not shown) strengthens the above speculation.

How does the pC194 replication origin function? pC194 replicates via a ss DNA intermediate (te Riele *et al.*, 1986a, b) and encodes a replication protein which introduces a nick (Michel and Ehrlich, 1986a) at the plasmid replication origin. It resembles in that respect the extensively studied *E.coli* ss DNA phages (Baas, 1985). Replication of these genomes is of the rolling circle type. One of the features of such replication is that it can terminate at a sequence homologous with the replication origin

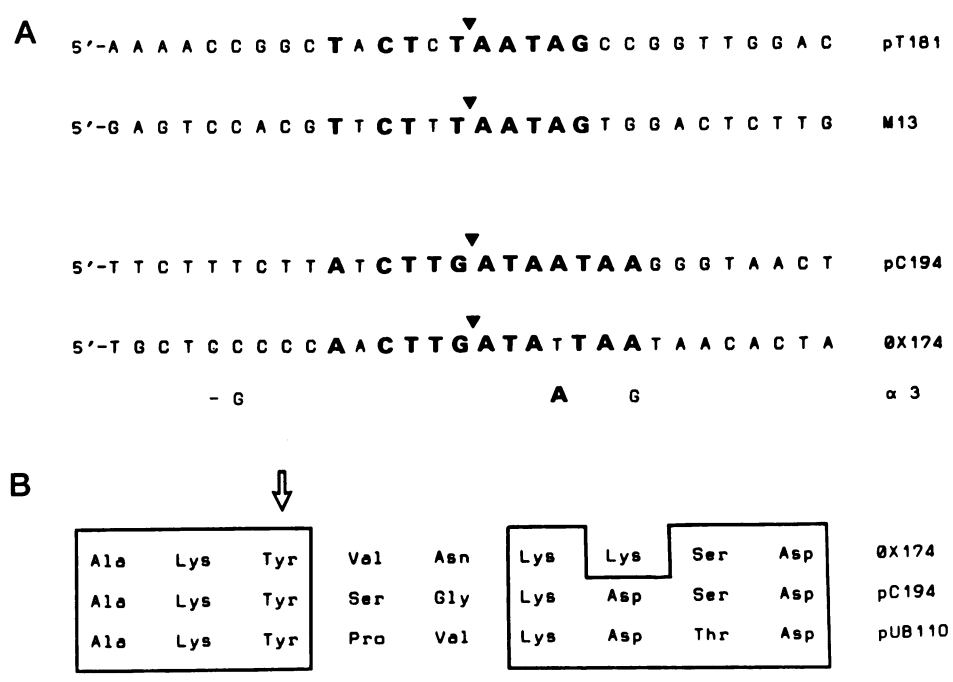

Fig. 5. (**A**) Homologies between plasmids and phages. Nucleotide sequence homology around the nick site (▼) of pT181 (Koepsel *et al.*, 1985) and M13 (Meyer *et al.*, 1979), and pC194 (Michel and Ehrlich 1986a) and ΦX174 (Langeveld *et al.*, 1978) or α3 (Heidekamp *et al.*, 1982); (**B**) aminoacid homology between the replication proteins of ΦX174 (van Mansveld *et al.*, 1986), pC194 (Horinouchi and Weisblum, 1982b) and pUB110 (McKenzie *et al.*, 1986). The tyrosine of the ΦX174 rep protein, covalently linked to DNA, is indicated by the arrow.

(Dotto *et al.*, 1982; Peeters *et al.*, 1987). We have shown here that pC194 replication can terminate at an 18 bp sequence homologous to the sequence which flanks the nick site in the plasmid replication origin. This result indicates that the pC194 replication origin functions in a way analogous to that of *E.coli* ss DNA phages and that the plasmid replicates as a rolling circle.

Comparison of the amount of plasmids generated by initiation of DNA synthesis at the pC194 origin and its termination at the homologous 18 bp sequence derived from pUB110 or synthesized *in vitro* suggests that the regions flanking the 18 bp sequence may affect the efficiency of termination. Further experiments are needed, however, to ascertain that point. It is interesting that DNA synthesis does not always terminate even at the complete plasmid replication origin, as judged from the fact that single-stranded plasmid multimers are detected (te Riele *et al.*, 1986a) On the other hand termination may occur, although with a low frequency, at sites resembling the smaller of the two origin hairpins (Michel and Ehrlich, 1986a; see above).

It is worth pointing out that many small, high copy number plasmids from Gram positive bacteria resemble pC194. The best characterized of these, pT181, replicates via a ss DNA intermediate (te Riele *et al.*, 1986a; Gruss *et al.*, 1987) and encodes a replication protein which nicks the plasmid replication origin (Koepsel *et al.*, 1985). A number of other plasmids, present in *Bacillus cereus, B. subtilis, S. aureus, Streptococcus lactis, Streptococcus pneumoniae* and *Streptomyces lividans* (te Riele *et al.*, 1986a, and personnal communications from M.Espinosa, H.Schrempf and W.de Vos) generate ss DNA and may therefore also replicate in a rolling circle mode. It is intriguing that so many plasmids from Gram positive bacteria adopted a life style which was previously observed only with certain bacteriophages from Gram negative bacteria.

Significant homology exists between DNA sequences flanking the site of the nick in plasmid pC194 and isometric *E.coli* phages (*α3*, ΦX174), and in plasmid pT181 and filamentous *E.coli* phages (M13, Figure 5A). We considered the possibility that the homology between the replication origins necessitates an homology between the regions of the proteins which recognize the replication origins. Comparison of amino-acid sequences of the ΦX174 and pC194 (and pUB110) replication proteins revealed the existence of a region of homology (Figure 5B). That region comprises the tyrosine of the ΦX174 protein which is covalently attached to DNA upon nicking of the replication origin (van Mansveld *et al.*, 1986). The observed homologies and the similar replication modes suggest that an evolutionary relationship exists between the ss DNA phages from Gram negative bacteria and the ss DNA plasmids from Gram positive bacteria.

Materials and methods

The bacterial strains used were *B.subtilis* 8G-5 (*his trp tyr ade ura nic rib*; S. Bron) and *E.coli* strains HVC45 (*lac leu thi ton str hsdR;* R. Davis) and HVC293 (*lac leu thi trp str hsdR recA;* lab collection).

The plasmids used in this study are listed in Table I. Figure 1 shows plasmid pHV33, composed of pBR322 and pC194, linked at their unique *Hin*dIII sites (Primrose and Ehrlich, 1981).

Deletion derivatives of pHV33 that had lost pC194 sequences carrying the Cm-resistance gene were obtained by Michel and Ehrlich (1986a,b) as follows: Plasmids consisting of phage M13mp2 or R229 (a phage f1 derivative) and pHV33 linked at the *Eco*RI site suffer from deletions in *E.coli*. Five derivatives which had lost most of the phage sequence and the pC194 sequence carrying the Cm-resistance gene were genetically labeled by inserting the pE194 Em-resistance gene (a *Sau*3A segment between nucleotide 1586 and 3186; Horinouchi and Weisblum, 1982a) in their *Bam*HI site. The plasmids which carried 74, 25, 5, 4 or 1 bp of the sequence upstream from the pC194 nick site were designated pHV33 Δ-74, Δ-25, Δ-5, Δ-4 and Δ-1, respectively.

Deletion derivatives of pHV33 that had lost pC194 sequences carrying the replication gene A were obtained *in vitro*: pHV33 Δ+21 resulted from deleting the segment between the pC194 *Nae*I site and the pBR322 *Pvu*II site (Figure 1). To obtain pHV33 Δ+40, a *Bam*HI linker sequence was inserted in the unique *Afl*II site of pHV33 (Figure 1), resulting in pHV1042 (see below). Deletion of the segment between this *Bam*HI site and the pBR322 *Pvu*II site gave plasmid pHV33 Δ+40. Plasmid pHV60 resulted from a spontaneous deletion in pHV14 (a plasmid analogous to pHV33; Ehrlich, 1978) of a segment between the start of the tetracycline resistance gene (position 39 of pBR322) and the nucleotide

located 2 bp downstream of the pC194 nick site (Michel and Ehrlich, 1986a; Primrose and Ehrlich, 1981).

pHV1020 was constructed as follows: a *Bam*HI linker was inserted in the unique *Hha*I site of pC194 (position 1513; numbering according to Dagert *et al.*, 1984), which gave the plasmid pHV1015. The segment between the *Bam*HI site and the *Pvu*II site at position 2592, carrying the replication gene A, was inserted into *Bam*HI/*Pvu*II cleaved pUB110 to give pHV1020.

Plasmids pHV1041 and pHV1042 resulted from mutating the unique *Afl*II site of pHV33 (located 40 bp downstream from the pC194 nick site, see Figure 1) as follows: pHV33 was digested with *Afl*II, the linearized plasmid was purified from a gel and treated with Klenow DNA polymerase I. DNA was then ligated in the absence or in the presence of a *Bam*HI linker and introduced into *E.coli*. Two plasmids lacking the *Afl*II site (one from each experiment) were analysed by sequencing. pHV1041 contained a TTA insertion at position 41 downstream from the pC194 nick site (see Figure 3B); pHV1042 contained the *Bam*HI linker sequence at position 40, but an A was deleted (see Figure 3C). pHV1045 was obtained by inverting pC194 in pHV1042.

Plasmids pHV1050 and pHV1053 were constructed by inserting into the *Bam*HI site of pHV15 (a plasmid composed of pBR322 and pC194 joined in the orientation opposite to that in pHV33; Ehrlich, 1978) and pHV33, respectively, the 900 bp *Sau*3A fragment C of pUB110 (McKenzie *et al.*, 1986). To construct pHV1055 and pHV1057, the 18 bp sequence corresponding to the region of the pC194 origin between nucleotides −15 and +3, relative to the nick site, flanked by a *Sal*I and a *Sma*I site was synthetized *in vitro*. It was inserted into pHV15 cleaved with *Sal*I and *Eco*RV or into pHV33 cleaved with *Sal*I and *Nru*I to yield pHV1055 and pHV1057, respectively.

In order to clone the pC194 replication origin, plasmid pHV1051 was constructed, by inserting the *Sau*3A segment C of pUB110 into pHV1045. This plasmid is similar to pHV1050, but contains the *Bam*HI linker at the *Afl*II site. It generated spontaneously in *E.coli* a smaller derivative, pHV1061, that contained a putative pC194 origin, flanked by an *Xmn*I site and the *Bam*HI linker sequence (see text for the discussion of that event) The *Xmn*I−*Bam*HI fragment of pHV1061 which carries the pC194 origin was inserted into *Sma*I/*Bam*HI cleaved pUC18. This plasmid was genetically labelled by replacing its *Hin*dIII−*Aat*II segment by a *Hin*dIII-*Aat*II fragment of plasmid pHV1025 (a pBR322 derivative containing in the *Cla*I site the pE194 *Taq*I segment A which carries the Em-resistance gene). The resulting plasmid was named pHV1062.

Plasmid DNA was extracted from *E.coli* by the cleared lysate method (Clewell and Helinski, 1969) and purified by hydroxyapatite chromatography (Colman *et al.*, 1978) and CsCl density centrifugation. For analytical purposes DNA was extracted from *E.coli* and *B.subtilis* by the alkaline lysis method (Birnboim and Doly, 1979). *E.coli* and *B.subtilis* were transformed by standard procedures (Niaudet and Ehrlich, 1979; Bron and Venema, 1972).

Smaller plasmids accompanying pHV1050, pHV1051 and pHV1055 were purified by gel electrophoresis and reintroduced into *E.coli*. DNA was extracted by the cleared lysate method and purified. The smaller plasmid accompanying pHV1053 was extracted from an agarose gel and purified using GeneClean (Bio 101, CA, USA). Their structure was determined by restriction and sequence analysis according to Maxam and Gilbert (1980).

Acknowledgements

We are grateful to B.Michel for the gift of pHV33 deletion derivatives. This work was supported by grants from the Ministère de la Recherche et de l'Enseignement Supérieur (510070 and 510138) and the European Economic Community (BAP-0141-F).

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Received on July 13, 1987