The EMBO Journal vol.8 no.9 pp.2711 – 2716, 1989

Replication origin of a single-stranded DNA plasmid pC194

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Communicated by D.Ehrlich

The replication of the single-stranded (ss) DNA plasmid pC194 by the rolling circle mechanism was investigated using chimeric plasmids that possess two pC194 replication origins. One of the origins was intact, whereas the other was either intact or mutated. The origins were activated by inducing synthesis of the pC194 replication protein, under the control of lambda phage p_L promoter. Initiation of pC194 replication at one origin and termination at the other generated circular ssDNA molecules smaller than the parental chimeric plasmid. From the nature and the amount of ssDNA circles, the activity of an origin could be assessed. Our results show that (i) the signal for initiation of pC194 replication is more stringent than that for termination; (ii) the sequence and structure of the origin are important for its activity and (iii) successful termination of one replication cycle is not followed by reinitiation of another. This last observation differentiates a ssDNA plasmid (pC194) from a ssDNA phage (ϕX174).

Key words: replication/ssDNA/pC194/plasmid

Introduction

Numerous plasmids from Gram-positive bacteria accumulate single-stranded (ss) DNA molecules as replication intermediates (te Riele *et al.*, 1986a,b). The first single-stranded plasmids studied were isolated from *Staphylococcus aureus* and *Bacillus subtilis*; similar plasmids were later identified in *Streptococcus* spp. (del Solar *et al.*, 1987; de Vos, 1987, and personal communication), *Streptomyces lividan* (Pigac *et al.*, 1988), *Clostridium butyricum* (N.Minton, personal communication), various *Staphylococcus* and *Bacillus* spp. (for a review, see Gruss and Ehrlich, 1989). Furthermore, there is evidence that ssDNA plasmids are present in hosts as distant as *Mycoplasma mycoides* (Bergmann *et al.*, 1989) and the archaebacteria *Halobacterium* (Sioud *et al.*, 1988).

The replication of two representative ssDNA plasmids, pC194 and pT181, has been studied in some detail. The following observations indicate that they replicate by a rolling circle type mechanism as described for *Escherichia coli* ssDNA phages (for a review, see Baas and Jansz, 1988). (i) Leading strand synthesis requires the presence of a plasmid-encoded replication protein which introduces a nick at a specific site in the plus-origin of the two plasmids (Koepsel *et al.*, 1985; Michel and Ehrlich, 1986). This generates a 3'OH end which is used as a primer for DNA synthesis. (ii) Termination of replication occurs within a subsequence of the plus-origin which contains the nick site (Gros *et al.*, 1987; Khan *et al.*, 1988; Iordanescu *et al.*, 1988). (iii) ssDNA molecules are converted to double-stranded (ds)DNA by complementary strand synthesis, often initiated at a specific site present on the ssDNA template (te Riele *et al.*, 1986b; Gruss *et al.*, 1987).

Stretches of homology found in the replication proteins and origins of different ssDNA plasmids allowed their ordering onto several plasmid families, one including pT181 and another pC194 (see Gruss and Ehrlich, 1989, for a review). Interestingly, the latter also includes the *E.coli* ssDNA phage ϕX174 (Gros *et al.*, 1987). These observations suggest that all the inter-related plasmids, and probably all ssDNA replicons replicate by the same basic

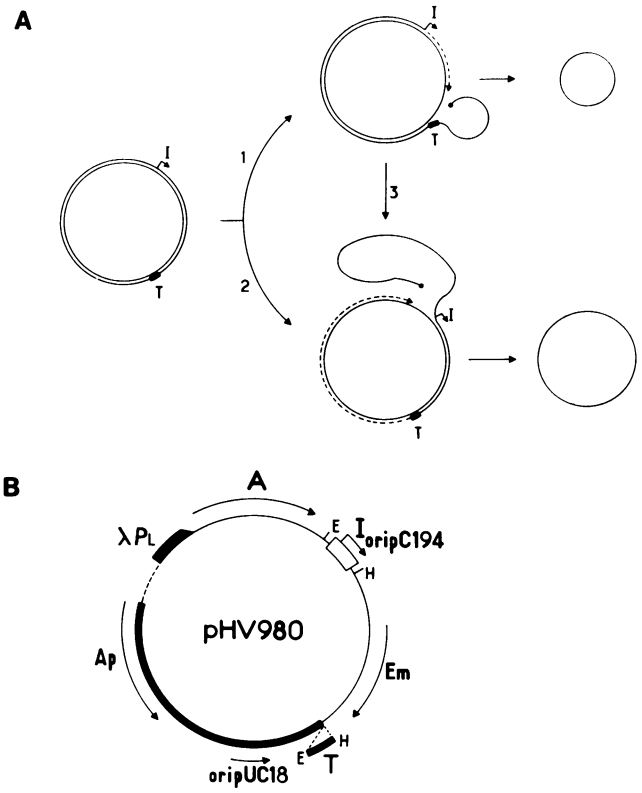

Fig. 1. Initiation and termination of rolling circle replication. (**A**) Schematic representation of the generation of single-stranded circles (see text for explanation). (**B**) Plasmid pHV980. The pC194 plus-origin is present at position I (box), the nick-site and the direction of replication is indicated by the arrow. The coding sequence A of the pC194 replication protein is transcribed from the thermo-inducible lambda p_L promoter. The thick line represents pUC18 sequences, the polylinker in which sequences to be tested for termination activity can be inserted is indicated by T. Genes conferring resistance to ampicillin (Ap) and erythromycin (Em), the pUC18 replication origin and restriction sites for *Eco*RI (E) and *Hin*dIII (H) are indicated.

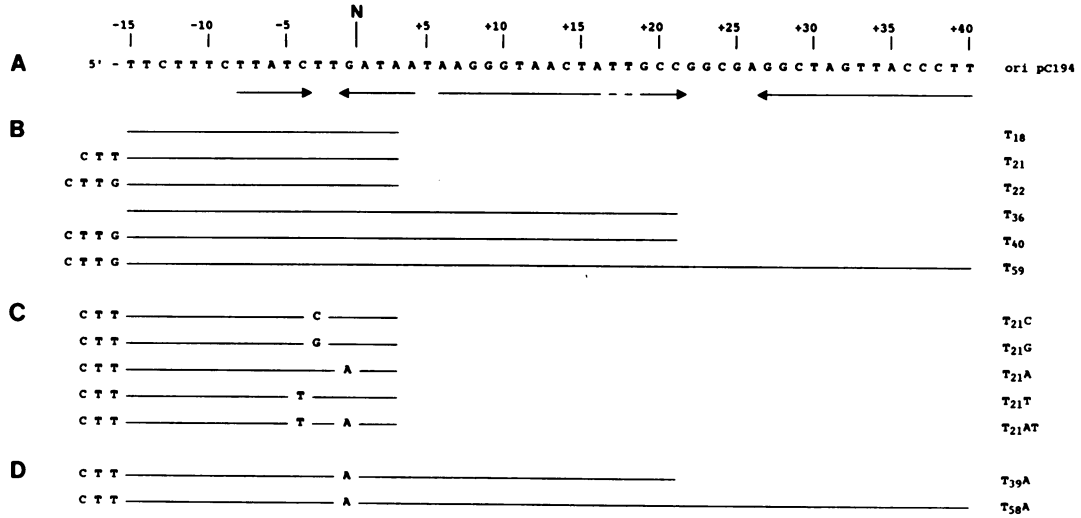

Fig. 2. Sequences related to pC194 replication origin. (**A**) The 55 bp complete origin sequence carried at I of the plasmid pHV980. N indicates the site nicked by the pC194 replication protein A upon initiation of rolling circle replication, that proceeds to the right. The arrows under the sequence indicate the small (5 bp) and large (14 bp) palindromes. Numbers refer to positions upstream (−) and downstream (+) of the nick-site.
(**B**)−(**D**) Origin sequences carried at T of plasmid pHV980. The lines represent sequences identical with the complete origin; differing nucleotides are indicated.

rolling circle mechanism. The implied generality of this mode of replication, and the fact that it was studied in detail only in *E.coli* ssDNA phages (see Baas and Jansz, 1988, for a review) led us to further characterize it in a ssDNA plasmid.

Results

Experimental strategy

Rolling circle replication of ssDNA phages and plasmids is initiated and terminated at the same site in the plus-origin of replication, giving ssDNA circles of replicon size. In chimeric molecules containing two such origins, replication can be initiated at one and terminated at the other (Dotto and Zinder, 1984; Gros *et al.*, 1987; Goetz and Hurwitz, 1988). Single-stranded DNA circles are thus generated, the size of which corresponds to the distance between the two origins.

In order to define the parameters that are important for the termination of pC194 replication, we used hybrid plasmids carrying two replication origins (Figure 1). One of the origins, denoted I, was intact, the other, denoted T, was either intact, truncated or/and mutated. Initiation of replication at I and termination at T should generate small ss circles (pathway 1, Figure 1A). If the termination sequence T is not fully active, and is 'missed' by the replication machinery, termination will occur at I, which will generate full-sized ssDNA circles. The efficiency of termination at T is therefore reflected in the ratio of the amount of small- and full-size ssDNA molecules.

The same plasmids were used to study the parameters important for initiation of pC194 replication. Initiation at T and termination at I should generate medium-sized circles (pathway 2, Figure 1A) and the efficiency of the process could be estimated from the ratio of the medium- to full- and small-sized circles.

Induction of rolling circle replication

To measure termination efficiency it is essential that ssDNA circles are detected shortly after being generated, prior to their conversion to dsDNA. For this purpose, we used an inducible system allowing detection of newly generated ssDNA. The experiments were carried out in *E.coli*, in which pC194 replicates efficiently (Goze and Ehrlich, 1980; te Riele *et al.*, 1986b). Plasmid constructions, related to plasmid pHV980 (Figure 1B), contained the following

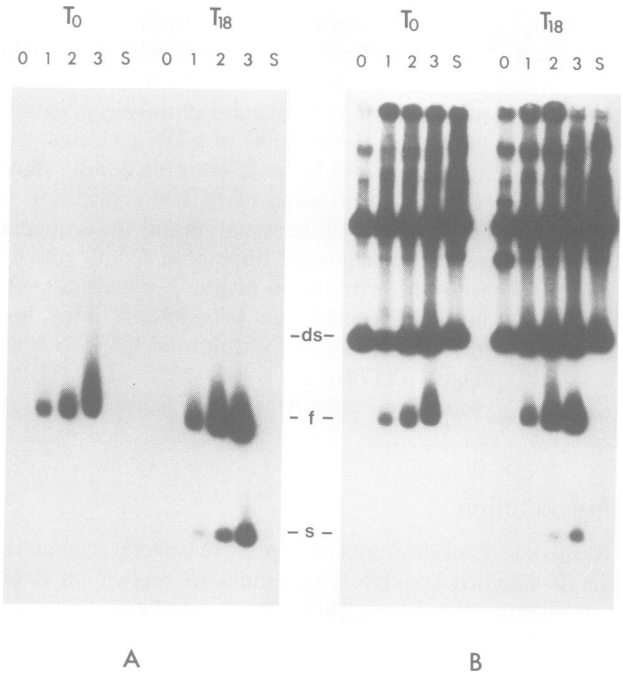

Fig. 3. Generation of single-stranded DNA upon induction of rolling circle replication. Lysates of cells containing plasmids pHV980T_0 and pHV980T_{18}, prepared after 0, 15, 30 and 60 min at 42°C (lanes 0, 1, 2 and 3, respectively), were electrophoresed through agarose gels and the DNA was transferred to nitrocellulose filters without (**A**) and after (**B**) denaturation. The 60-min lysate was also treated with nuclease S1 and run in lane S. Filters were hybridized with the probe containing the erythromycin resistance gene to detect full- (f) and small-(s) sized ssDNA circles. ds indicates the position of the parental double-stranded monomer.

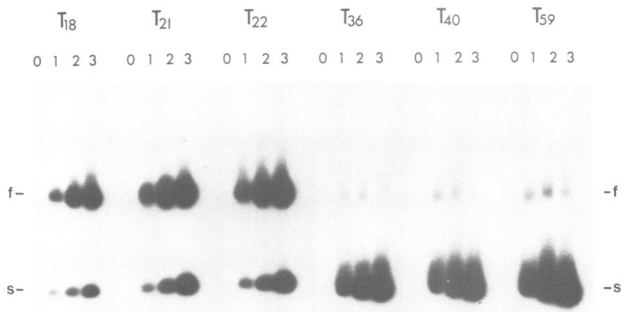

Fig. 4. Length requirement for termination. Rolling circle replication was initiated in pHV980 derivatives containing the sequences T_{18} to T_{59} (cf. Figure 2B). Filters were hybridized to the probe containing erythromycin resistance gene to detect full- (f) and small- (s) sized ssDNA circles, as described in the legend to Figure 3A.

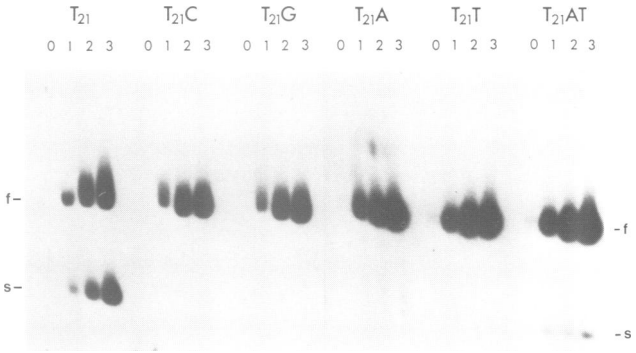

Fig. 5. Sequence requirement for termination. Rolling circle replication was initiated in pHV980 derivatives containing the wild-type or the mutated T_{21} sequences shown in Figure 2C. Filters were hybridized to the probe containing the erythromycin resistance gene to detect full- (f) and small- (s) sized ss DNA circles as described in the legend to Figure 3A.

elements: (i) the replication functions and ampicillin resistance gene of pUC18, which ensure maintenance of the plasmid in *E.coli*; (ii) the coding sequence of the pC194 replication protein A, placed behind the lambda p_L promoter which is controlled by the thermosensitive lambda *c*I857 repressor; (iii) the 55 bp origin sequence of pC194 which contains the entire replication origin (I, Figure 2A); (iv) the pUC18 polylinker, separated from the pC194 origin by an erythromycin resistance gene. Various termination signals were inserted into the polylinker; they were designated T_n, where '*n*' represents the length of the inserted sequence (in bp). The lambda repressor gene was carried either on a prophage or on plasmid pHV983, which is compatible with pHV980 (see Materials and methods).

At non-permissive conditions for synthesis of the pC194 replication protein (28°C), plasmid pHV980 did not generate ssDNA (Figure 3A, T_0, lane 0). Induction of synthesis of the pC194 replication protein by raising the temperature to 42°C, resulted in the appearance of ssDNA (Figure 3A, T_0, lanes 1–3). The ss nature of this material was confirmed by its sensitivity to S1 nuclease (lane 5).

Dependence of termination efficiency on the length of the termination signal

We determined termination at five pC194 sequences, ranging from 59 bp, which corresponds to the complete pC194 origin, to 18 bp which includes the nick-site (Figure 2B, T_{59}, T_{40}, T_{36}, T_{21}, T_{18}). In addition, a 22 bp pUB110 sequence, differing by 1 bp from the pC194 sequence (Figure 2B, T_{22}) was also tested. The plasmid containing the longest sequence generated predominantly small-sized ssDNA, accompanied by very little full-sized ssDNA; medium-sized ssDNA, expected from initiation at T_{59} and termination at I was not detected with the probe used (Figure 4, T_{59}).

The small-sized ssDNA molecules generated by initiation at I and a successful termination at T should be circular and carry a pC194 replication origin flanked by two *Hin*dIII sites (cf. Figure 1B) 123 bp apart. This DNA was purified, converted into a ds form by *in vitro* DNA synthesis and cleaved by *Hin*dIII. Only a 123 bp segment was detected (not shown) which indicates that the small-sized ssDNA molecules were circular and that the termination was therefore successful.

No conversion of ss to dsDNA occurred during the experiment, as judged by the absence of small ds circles (Figure 3B, T_{18}). Conversion therefore did not affect measurement of termination efficiency.

Sequences of 40 and 36 bp had a termination activity comparable to that of the full origin (Figure 4, T_{36}, T_{40} and T_{59}). In contrast, the two shorter pC194 sequences as well as the 22 bp pUC110 sequence were less active, since the small ssDNA represented only ~20% of the total ssDNA (Figure 4). These results show that fully efficient termination occurs on a 36 bp sequence, starting 15 bp upstream from the nick and ending 21 bp downstream of it (Figure 2B, T_{36}), whereas only inefficient termination occurs on a 21 bp sequence, starting at the same site but ending 5 bp downstream of the nick (Figure 2B, T_{21}). The 1 bp difference between the pUB110 and the pC194 sequences (Figure 2B, T_{22}) did not affect the efficiency of pC194 termination.

Sequence requirement for termination

The pC194 origin sequence contains two palindromes, 14 and 5 bp long, the latter containing the nick-site (Figure 2A). The larger palindrome is not necessary for efficiency termination, since the 36 bp sequence lacking it (Figure 2B, T_{36}) had a maximal termination activity. The importance of the smaller palindrome was investigated by introducing point mutations in the 21 bp sequence (Figure 2B, T_{21}). First, the centre of the small palindrome, corresponding to two T residues, was mutated by changing the T at position −3 to a C or a G (Figure 2C, $T_{21}C$, $T_{21}G$). Second, the complementarity of the sequence flanking the two central T residues was modified by replacing the C at position −4 by a T or the G at position −1 by an A (Figure 2C, $T_{21}T$, $T_{21}A$). The latter replacement also changed the sequence of the nick-site. Finally, by changing the motif CTTG into TTTA, a double mutation was introduced, which maintained complementarity but modified the sequence at the nick-site (Figure 2C, $T_{21}AT$). All the single base substitutions completely abolished termination (Figure 5). Strikingly, a double mutation, which maintained sequence complementarity around the two T residues ($T_{21}AT$), restored termination activity to a low but detectable level.

The wild-type 21 bp sequence did not possess full termination activity. To determine the effect of a mutation in a fully active sequence, we replaced the G at position −1 by an A in a 39 and a 58 bp sequence (Figure 2D, $T_{39}A$, $T_{58}A$). Figure 6A shows that no termination took place at

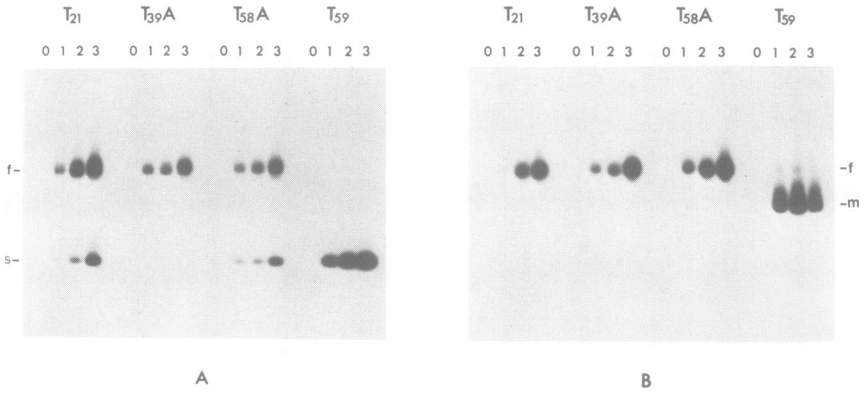

Fig. 6. Effect of mutations on termination and initiation. Rolling circle replication was initiated in pHV980 derivatives containing the indicated sequence. Single-stranded DNA was detected as described in the legend tφ Figure 3A. (**A**) Hybridization to the probe containing the erythromycin resistance gene to detect full- (f) and small- (s) sized ssDNA circles (measuring initiation at I and termination at T). (**B**) Hybridization to the probe containing the ampicillin resistance gene to detect full- (f) and medium- (m) sized ssDNA circles (measuring initiation at T and termination at I).

the shorter of the two mutated sequences ($T_{39}A$). Surprisingly, termination did occur at the longer ($T_{58}A$), albeit at a lower rate than that observed with the wild-type origin sequence. Homologous recombination did not contribute to this process since similar amounts of ssDNA were found in a *recA*$^+$ and a *recA* strain (HVC293 was used, data not shown). Apparently, the presence of sequences extending beyond position +21 can partially suppress the effect of a mutation that is deleterious for termination at a shorter sequence.

Sequence requirement for initiation of rolling circle replication

Initiation of rolling circle replication at sequences inserted in the polylinker T of pHV980, and termination at the pC194 plus-origin, would generate medium-sized ssDNA circles. These could be detected with a probe containing the ampicillin resistance gene, which would not hybridize with the small-sized ss circles (Figure 1). We tested this possibility for the wild-type sequences of 59 and 21 bp and the mutated sequences of 58 and 39 bp in which the G at −1 was replaced by an A. As expected, a large amount of medium-sized ssDNA was generated upon induction when a 59 bp sequence was present at T (Figure 6B, T_{59}). In contrast, no medium-sized ssDNA was detected with the 21 bp wild-type sequence (T_{21}) or the two mutated sequences ($T_{39}A$, $T_{58}A$), which indicates that no initiation occurred at these sequences. Since two of these (T_{21}, $T_{58}A$) had a substantial termination activity (see Figure 6A), the sequence requirements for initiation are apparently more stringent than for termination. A second conclusion that can be drawn from this experiment is that successful termination at the truncated or the mutated origin sequence is not followed by reinitiation of DNA synthesis.

Discussion

The complete replication origin of ssDNA plasmid pC194 is carried on a 55 bp segment; however, shorter segments have partial origin activity (Gros *et al.*, 1987). To measure this activity we constructed pUC18-derived plasmids carrying the pC194 replication origin which could be conditionally activated. *E.coli* was used as host, since pC194 can replicate in this bacterium (Goze and Ehrlich, 1980; te Riele *et al.*, 1986b). An attractive aspect of the conditional system was that it allowed detection of origin activities ranging from full to very low.

Three features of pC194 replication emerged from these studies. First, initiation requires a more stringent signal than termination. The complete wild-type 55 bp origin was necessary for initiation, while termination occurred with full efficiency at a shortened 36 bp origin, and to some extent even at the 18 bp sequence flanking the nick site as well as at the complete mutated origin sequence.

Second, both the sequence and the structure of the origin affect termination. In the wild-type origin the nick site is contained within a sequence which can be folded into a hairpin with a 4 bp stem and 2 base loop (Figure 2A). Two types of single base changes abolished termination. The first modified the loop of the putative hairpin, and therefore changed the sequence but not the structure of the origin. The second modified the stem, and changed both the sequence and the structure. Interestingly, a double mutation which preserved the complementarity of the stem also preserved some termination activity. Previous observations suggested that short sequences related to the doubly mutated origin might be recognized by the pC194 replication protein (Michel and Ehrlich, 1986). It was shown *in vitro* that the hairpin structures have predominantly 2 base loops when the last base pair of the stem is G:C and 4 base loops, when it is A:T (Orbons *et al.*, 1986). In the wild-type origin this last base pair is G:C, whereas in the double mutant it is A:T (Figure 2). The activity of the origin might be related to the efficiency of formation of the hairpin structure with 2 base loops. Sequences distal from the nick-site are also important for termination, since a single base change in the 21 and 39 bp signal abolished termination, while the same change in a 58 bp signal only reduced but did not eliminate termination activity. The role of these distant sequences is not understood at present.

The third feature of pC194 replication revealed in this study is that reinitiation does not follow successful termination. In phage φX174, termination of one round of replication is coupled with the initiation of another (Roth *et al.*, 1982; Brown *et al.*, 1984; Goetz *et al.*, 1988a,b). For example, Goetz *et al.* (1988a,b) have shown that a 28 bp phage sequence had a full initiation and termination activity while a 26 bp sequence had no initiation and ~20% of the

full termination activity. Strikingly, in chimeric plasmids having one complete (28 bp) and one truncated (26 bp) phage origin, replication initiated at the first origin and terminated at the second was always reinitiated at the second origin (Figure 1, pathway 3). In contrast, termination at truncated or mutated pC194 origins was never followed by reinitiation in our studies. The difference between ϕX174 and pC194, which belong to the same family of ssDNA replicons (Gros *et al.*, 1987) may reflect adaptive differences between a virulent phage and a plasmid. The phage life cycle requires production of a high number of DNA copies in a short time, irrespective of the fate of its host, whereas the plasmid has to control its copy number to preserve the host. It is known that the copy number of another ssDNA plasmid, pT181, is controlled by a tight regulation of the synthesis of the replication protein (Novick *et al.*, 1984). A prerequisite for such regulation might be the inability of the plasmid replication protein to reinitiate a novel round of replication upon termination of the preceding one.

Materials and methods

Bacteria

The following *E.coli* strains were used for the construction of plasmids: MC1061 (λ) *hsdR hsdM*$^+$ *hsdS*$^+$ *araD*139Δ (ara−leu)7697Δ (*lac*)X74 *galU galK rpsL*(str^r) (kindly provided by E.Remaut), which expresses the lambda *c*I repressor constitutively; JM105 Δ(*lac*−*proAB*) *thi rspL*(*str*^{r}) *endA sbcB supE hsdR/F'* [*traD*36 *proA*$^+$ *proB*$^+$*lacI*^{q} *lacZ*ΔM15]. The replication experiments were performed in *E.coli* strains K12ΔH1Δtrp (Remaut *et al.*, 1981) expressing the thermolabile lambda cI857 repressor and HVC293 *lacY*1 *leu*-6 *thrA*1 Δ*trpE*5 *hsdR recA*1 str^r (laboratory collection).

Plasmids

Plasmid pHV980 (Figure 1B) consists of five segments. (i) The pUC18 sequence containing the ampicillin resistance gene, pBR322 replication origin and the synthetic polylinker. (ii) A *Pvu*I−*Bam*HI segment containing the lambda pL promoter and a short preceding sequence, derived from plasmid pLC28 (Remaut *et al.*, 1981). (iii) Downstream of the promoter, the *Rsa*I−*Pvu*II segment of pC194 (nucleotides 1533−2592, Dagert *et al.*, 1984) which contains the coding sequence A of the replication protein but not the promoter (unpublished results). (iv) The 55 bp pC194 origin region (bp 1430−1485, according to numbering by Dagert *et al.*, 1984). (v) The erythromycin resistance gene of pE194 (bp 1940−3383 according to numbering by Horinouchi and Weisblum, 1982a,b), derived from plasmid pHV1062 (Gros *et al.*, 1987).

Sequences to be tested for termination activity were synthesized *in vitro* together with the flanking *Sma*I and *Sal*I sites and inserted into the matching sites of the pUC18 polylinker. They were then transferred from these plasmids into pHV980 by exchanging the segment bordered by the *Hin*dIII site of the polylinker and the *Pvu*I site of the ampicillin resistance gene. Plasmid pHV983 contains the p15A replicon and a kanamycin resistance gene, and expresses the thermolabile lambda repressor cI857. It corresponds to the *Bam*HI−*Eco*RI segment of pGP1-2 (Tabor and Richardson, 1985).

Induction of rolling circle replication

Plasmids pHV980 and its derivatives were introduced into *E.coli* strain K12ΔH1Δ*trp* or, together with pHV983, into HVC293 (*rec*A). Rolling circle replication was induced as follows: cultures were grown at 28°C in LB medium supplemented with ampicillin to an OD_{650} of 0.4; a sample was taken and chilled on ice (t = 0), while the remainder of the culture was transferred to 42°C to activate the lambda p_L promoter. Samples were withdrawn after 15, 30 and 60 min and chilled on ice. The OD_{650} of each sample was measured and lysates were prepared from culture volumes containing equal amounts of cells. The lysates were extracted with phenol and treated with RNAse I as described previously (te Riele *et al.*, 1986a).

Detection of ssDNA

Lysates were electrophoresed as described (te Riele *et al.*, 1986a) and DNA was transferred to nitrocellulose by vacuum blotting (LKB) without or after denaturation. Radioactive probes were prepared by nick-translation of pE194 or a plasmid containing the pBR322 sequence between the *Hin*dIII and *Pvu*II sites. The two probes detect the erythromycin and the ampicillin resistance genes of pHV980, respectively.

Analysis of small-sized ssDNA

K12ΔH1Δtrp cells carrying the pHV980T_{36} plasmid were incubated for 60 min at 42°C and lysed. The lysates were adjusted to a density of 1.8 g/ml by addition of caesium chloride and centrifuged for 4 h in a TL-100 rotor. The fractions enriched in ssDNA were dialysed and adjusted to 3−4 M with NaI. Double-stranded molecules were bound to silica particles (Geneclean Kit BIO101), which were removed by low speed centrifugation. The supernatant was dialysed against 10 mM Tris/1 mM EDTA and DNA recovered by ethanol precipitation. This was ssDNA, since it was sensitive to nuclease S1 and bound to nitrocellulose without denaturation. It was used as a template for complementary strand synthesis, which was primed upstream of the erythromycin resistance gene and proceeded in the direction of the pC194 origin (Figure 1). Cleavage of the resulting dsDNA with *Hin*dIII released a 123 bp segment which is expected if the small-sized ssDNA was circular and generated by initiation at I and termination at T.

Acknowledgements

We are grateful to E.Remaut for the gift of *E.coli* strains MC1061 (λ) and K12ΔH1Δtrp, M.A.Petit for help with some experiments and C.Anagnostopoulos, L.Jannière and A.Gruss for critical reading of the manuscript. This work was supported by grants from the European Economic Community (BAP-0141-F) and the Fondation pour la Recherche Médicale.

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Received on May 2, 1989; revised on June 9, 1989