Plasmid rolling circle replication: identification of the RNA polymerase-directed primer RNA and requirement for DNA polymerase I for lagging strand synthesis

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Plasmid rolling circle replication involves generation of single-stranded DNA (ssDNA) intermediates. ssDNA released after leading strand synthesis is converted to a double-stranded form using solely host proteins. Most plasmids that replicate by the rolling circle mode contain palindromic sequences that act as the single strand origin, *sso*. We have investigated the host requirements for the functionality of one such sequence, *ssoA*, from the streptococcal plasmid pLS1. We used a new cell-free replication system from *Streptococcus pneumoniae* to investigate whether host DNA polymerase I was required for lagging strand synthesis. Extracts from DNA polymerase I-deficient cells failed to replicate, but this was corrected by adding purified DNA polymerase I. Efficient DNA synthesis from the pLS1-*ssoA* required the entire DNA polymerase I (polymerase and 5′–3′ exonuclease activities). ssDNA containing the pLS1-*ssoA* was a substrate for specific RNA polymerase binding and a template for RNA polymerase-directed synthesis of a 20 nucleotide RNA primer. We constructed mutations in two highly conserved regions within the *ssoA*: a six nucleotide conserved sequence and the recombination site B. Our results show that the former seemed to function as a terminator for primer RNA synthesis, while the latter may be a binding site for RNA polymerase.
Keywords: DNA polymerase I/lagging strand synthesis/ primer RNA/RNA polymerase/rolling circle replication

Introduction

Plasmid rolling circle (RC) replication is an asymmetric mechanism in which termination of leading strand synthesis results in generation of single-stranded DNA (ssDNA) molecules (te Riele *et al.*, 1986; Murray *et al.*, 1989). These intermediates are the hallmark of a number of small multicopy plasmids, generically termed RCR plasmids, whose mechanism of replication resembles that of ssDNA coliphages (reviewed in Zinder and Horiuchi, 1985; Baas and Jansz, 1988; Gruss and Ehrlich, 1989; Novick, 1989; del Solar *et al.*, 1993a; Khan, 1996). Replication of the plasmid lagging strand is mediated by host factors and initiates from a highly structured non-coding region termed the plasmid single strand origin, *sso*

(del Solar *et al.*, 1987a; Gruss *et al.*, 1987). It has been postulated that lagging strand synthesis is initiated by a primer RNA (pRNA) which is synthesized from the *sso* (Birch and Khan, 1992; Dempsey *et al.*, 1995). Primer synthesis is thought to be performed by the host RNA polymerase (RNAP) since the presence of the RNAP inhibitor rifampicin leads to: (i) *in vivo* accumulation of ssDNA (Boe *et al.*, 1989; Kramer *et al.*, 1995); (ii) inhibition of *in vitro* DNA replication of the RCR plasmid pLS1 (del Solar *et al.*, 1987b); and (iii) inhibition of *in vitro* DNA synthesis from ssDNA templates prepared from various RCR plasmids (Birch and Khan, 1992; Dempsey *et al.*, 1995). However, based on either sequence homologies or partial inhibition by rifampicin, it has been proposed that lagging strand replication may also be primed by the host DNA primase or by a φX174-type primosome (Boe *et al.*, 1991; Leenhouts *et al.*, 1991; Seegers *et al.*, 1995). Following pRNA synthesis, host DNA polymerases would accomplish the single-stranded-→double-stranded plasmid DNA conversion (Díaz *et al.*, 1994).

Sequence homologies and functional analyses have revealed the existence of four types of *sso* in RCR plasmids: (i) *ssoA*, present in several staphylococcal replicons like pT181, pC221 and pE194 (del Solar *et al.*, 1987a; Gruss *et al.*, 1987); (ii) *ssoU* described for pUB110 (van der Lelie *et al.*, 1989); (iii) *ssoT* most commonly found in *Bacillus* plasmids (Bron *et al.,* 1987); and (iv) *ssoW*, recently described on the lactococcal plasmid pWV01, and proposed to exist in closely related replicons (Seegers *et al.*, 1995). The RCR plasmid pMV158 from the Gram-positive bacterium *Streptococcus*, contains both *ssoA* and *ssoU*, the latter being absent in its derivative plasmid pLS1 (Lacks *et al.*, 1986; Priebe and Lacks, 1989; van der Lelie *et al.*, 1989). In general, most *sso*s are fully functional only in their original, or closely related, hosts, with the exception of *ssoU* which appears to be functional in several Gram-positive species (Kramer *et al.*, 1995; Meijer *et al.*, 1995a,b). Whereas synthesis of the lagging strand from the pUB110-*ssoU* starts from a unique site, several initiation points seem to exist within the *ssoA* of pT181, pSN2 and pE194 (Dempsey *et al.*, 1995). Within the *ssoA* of different plasmids, two conserved sequences have been reported as being important for functionality (Novick, 1989): (i) the recombination site B (RS_B), involved in inter-plasmid recombination (Novick *et al.*, 1984), and proposed to play an important role in *ssoA* activity (Gruss *et al.*, 1987); and (ii) a 6 bp consensus sequence (5′-TAGCGT-3′, hereafter referred to as CS-6) located within the terminal loop of the major secondary structure of *ssoA* (del Solar *et al.*, 1987a). The existence of the CS-6 sequence within palindromic regions has been taken as indicative of the presence of *ssoA* in several plasmids (Zaman *et al.*, 1993). In the case of the pLS1-

ssoA, small changes within the CS-6 lead to a 2-fold increase in the amount of intracellular ssDNA accumulated in *Streptococcus pneumoniae*, suggesting a role for this sequence in the ss→dsDNA conversion (Kramer *et al.*, 1995).

In the present study, we have analysed the host functions involved in initiation of lagging strand synthesis. We have also investigated the role of the RS_B and CS-6 conserved regions on lagging strand replication from the plasmid pLS1-*ssoA*. Since this origin is fully functional in *S.pneumoniae*, we have developed an *in vitro* replication system from this bacterium. Employment of this system allowed us to determine the initiation points of DNA synthesis and to assess the role of the host-encoded DNA polymerase I (Spn PolI), which we show is essential for lagging strand replication. In addition, we demonstrate that, *in vitro*, RNAP from the Gram-positive bacterium *Bacillus subtilis* specifically binds to ssDNA harbouring the pLS1-*ssoA*, and synthesizes a 20 nucleotide pRNA from this template DNA. We have determined the 5′ and 3′ ends of the pRNA, and we show that the pRNA is elongated by the Spn PolI enzyme. To define the role of the conserved RS_B and CS-6 sequences, we have generated a collection of mutations within the above two regions of the pLS1-*ssoA*, and assayed pRNA synthesis. Our results show that whereas the RS_B acts as the primary site for RNAP binding, the CS-6 acts as a terminator of the pRNA synthesis. Our findings constitute the first direct evidence for the existence of a pRNA and clarify the role of the conserved sequences in lagging strand initiation in plasmids replicating by the RC mode.

Results

Features of the pLS1-ssoA
Plasmid pLS1 (4408 bp) was constructed by deletion of a 1128 bp *Eco*RI fragment from the natural plasmid pMV158 (Lacks *et al.*, 1986). This deletion removes the *ssoU* and the 5′ region of the *mobM* gene (Priebe and Lacks, 1989; Guzmán and Espinosa, 1997). We have localized the functional *ssoA* of pLS1 within a large palindromic non-coding region (del Solar *et al.*, 1987a; Kramer *et al.*, 1995), which is bordered by restriction sites *Afl*II and *Nco*I (Figure 1A). By deletion analyses, the pLS1-*ssoA* region has been defined between coordinates 4022 and 4221 (Kramer *et al.*, 1995), which includes the conserved RS_B and CS-6 sequences (Figure 1B). In addition to these sequences, two sets of short tandem repeats (repeat a, 5′-GCCGA-3′, and repeat b, 5′-ACGGAC-3′) flank the CS-6, but their relevance for *ssoA* activity is not yet clear (Figure 1B). Computer predictions of probable secondary structures generated within the *ssoA* (between coordinates 4086 and 4233) indicate that some nucleotides of the RS_B and of the CS-6 sequences are unpaired (see Figure 8). Since the activity of the *ssoA* is orientation dependent (Gruss *et al.*, 1987), unpaired sequences are believed to be relevant for plasmid lagging strand synthesis (del Solar *et al.*, 1993b; see below).

In vitro replication from pLS1-ssoA in S.pneumoniae
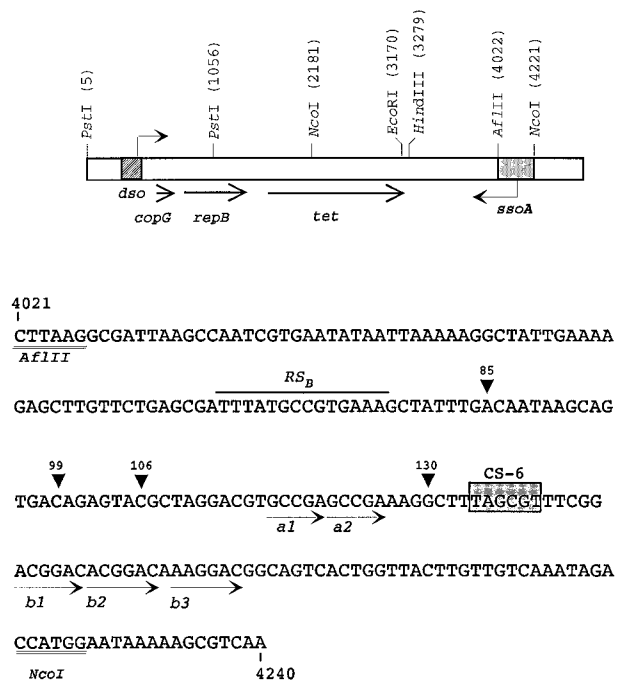Cell-free bacterial systems able to support replication of plasmids using the RC mode have been prepared from

Fig. 1. Features of plasmid pLS1 and its *ssoA* region. (**A**) Schematic map of pLS1 showing the plasmid regions (shaded) involved in leading (*dso*) and lagging (*ssoA*) strand synthesis (arrows). Plasmid-encoded genes (thick arrows) and relevant restriction sites are shown. (**B**) Nucleotide sequence of pLS1 between coordinates 4021 and 4240 (Lacks *et al.*, 1986). The conserved 6 bp consensus (CS-6) and the RS_B sequences are indicated. Direct repeats a and b and the initiation sites (vertical arrowheads) of lagging strand replication (see Figure 2C) observed in *S.pneumoniae* are indicated. Restriction sites *Afl*II and *Nco*I which define the pLS1-*ssoA* (Kramer *et al.*, 1995; doubly underlined) are shown.

Escherichia coli (del Solar *et al.*, 1987b) and from *Staphylococcus aureus* (Birch and Khan, 1992, and references therein). However, the functionality of the pLS1-*ssoA* in these two bacteria is poor (del Solar *et al.*, 1987a, and our unpublished results). This contrasts with *S.pneumoniae*, in which the pLS1-*ssoA* is an efficient ss→dsDNA conversion signal *in vivo* (del Solar *et al.*, 1987a). Consequently, to test the ability of the pLS1-*ssoA* in directing *in vitro* lagging strand replication, it was important to develop cell-free extracts from *S.pneumoniae*. To this end, the *Hin*dIII–*Pst*I fragment from pLS1 (co-ordinates 3279 to 5; Figure 1A) was cloned into the phagemid pALTER-1 to construct the recombinant pApLS1ssoA, which harbours the pLS1-*ssoA* in the functional orientation (Table I). In addition, the *Eco*RI–*Pst*I fragment (coordinates 3170 to 5; Figure 1A) was cloned in the same vector. This construction (pApLS1ssoA⁻) places the pLS1-*ssoA* in the non-functional orientation. The recombinants were used to prepare ssDNA, which were employed as templates. Synthesis of DNA in the pneumococcal extracts was determined by analysis of total labelled DNA as a function of the incubation time, using ssDNA from pApLS1ssoA as a template. The results showed that completion of the process (i.e. synthesis of full-length dsDNA molecules) was achieved after 60 min of incubation (Figure 2A). At shorter incubation times, partially replicated intermediates were observed. These results agree with previous observations performed for a set of RCR plasmids in *S.aureus* extracts (Dempsey *et al.*,

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Table I. Bacterial strains and plasmids used in this work

Bacteria	Relevant features		*polA* gene product	Reference
S.pneumoniae 708	*polA⁺ malM*		PolI	Lacks *et al.*, 1986
S.pneumoniae MP560	*polAΔ*(184–805 bp)Ω(81 bp::*cat*)		PolIc269	Díaz *et al.*, 1992
	Ω[184 bp::pET-3b(591–511 bp)]		PolIn351b	
	*malM*Ω{418 bp::φ[*polA'*(1054 bp)-*erm*]}			
S.pneumoniae MP547	*polA*Ω(1052 bp::*cat*)		PolIn351a	A.Díaz and P.López, unpublished
E.coli JM109	F'*recA*1		–	Sambrook *et al.*, 1989

| Plasmid	Size (bp)	Relevant properties in the *ssoA* region	Source or reference
pLS1	4408	wild type	Lacks *et al.*, 1986
pALTER-1 (pA)	5680	phagemid vector	Promega
pApLS1ssoA	6806	*Hin*dIII–*Pst*I (pLS1) cloned in pALTER digested with same enzymes; *ssoA* in functional orientation	this work
pApLS1ssoA⁻	6880	*Eco*RI–*Pst*I (pLS1) cloned in pALTER digested with same enzymes; *ssoA* in non-functional orientation	this work
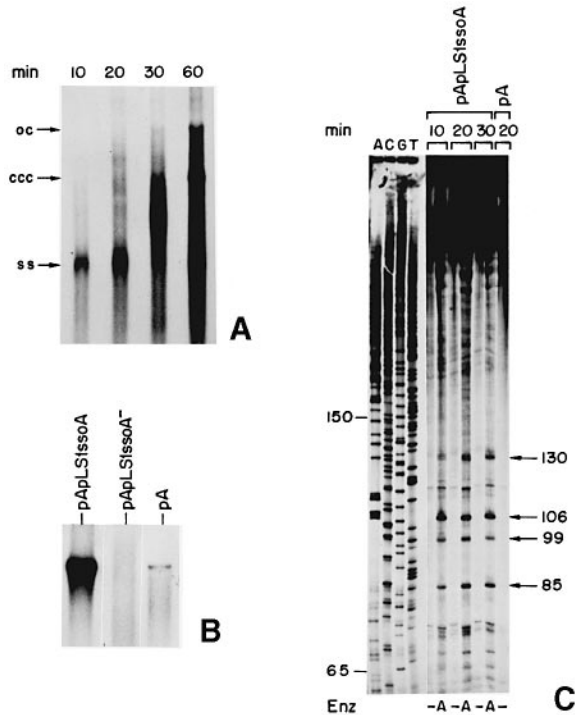
Fig. 2. Lagging strand replication in cell-free extracts from *S.pneumoniae*. (**A**) Time course experiments. ssDNAs containing the pLS1-*ssoA* in the functional orientation were replicated *in vitro* as described in Materials and methods, and the reaction products were analysed on 1% agarose gels without previous linearization. The position of the various forms of DNA is indicated: SS, single-stranded; CCC, covalently closed supercoiled; OC, double-stranded nicked open circles. (**B**) Replication of ssDNAs in pneumococcal extracts. ssDNAs from the phagemid pALTER-1 vector (pA) and from phagemids harbouring the pLS1-*ssoA* in the functional (pApLS1ssoA) or non-functional (pApLS1ssoA⁻) orientation were used. The reaction products were linearized with *Hin*dIII before electrophoresis on a 1% agarose gel. (**C**) Localization of the start sites of lagging strand synthesis from pLS1-*ssoA*. ssDNAs were obtained from pApLS1ssoA or from the vector pALTER-1 (pA). The template ssDNAs were allowed to replicate for the indicated times. Reaction products were digested with *Afl*II (A) or not digested (–) prior to their separation on a denaturing 8% polyacrylamide–urea gel. ACGT, sequencing ladder generated by the Sanger method (Sambrook *et al.*, 1989) used as size markers. Numbers indicate sizes in nucleotides.

1995). To avoid the smear caused by the different dsDNA forms (Figure 2A), total DNAs in the 60 min samples were isolated and linearized with *Hin*dIII, because its recognition site is placed at a position 3′-distal from the initiation of dsDNA synthesis. Thus, a single band of uniform size, corresponding to totally replicated dsDNA, was obtained (Figure 2B). No DNA synthesis was observed when the template ssDNAs were prepared from the vector (pA) or from the recombinant harbouring the pLS1-*ssoA* in the non-functional orientation (Figure 2B). *In vitro* DNA synthesis required RNAP-dependent synthesis of (an) RNA primer(s), since the process was inhibited by rifampicin or by lack of rNTPs (not shown). From these results, we conclude that pneumococcal extracts are able to support specifically *in vitro* replication of ssDNA from the pLS1-*ssoA* sequences.

To obtain information on the specificity of the *in vitro* initiation process, we determined the start site(s) of lagging strand synthesis in the *S.pneumoniae* cell-free extracts. This was performed through a time course experiment, using as a control ssDNA from the vector. Partially replicated pLS1-ssDNA molecules (from 10 to 30 min incubation times; Figure 2C) were either treated or not treated with *Afl*II. This enzyme cleaves downstream of the expected initiation sites of DNA replication from the *ssoA* (Figure 1A), and denaturation of the DNA samples should release replication products whose size will correspond to the distance between the initiation sites of lagging strand synthesis and the enzyme cleavage site. The replication products from undigested DNA samples consisted mainly of a series of large molecules (Figure 2C). These samples were taken as controls, because they allowed us to distinguish the specific replication products, which will be those observed only in the digested samples. In the case of the *Afl*II-digested DNA samples, three major bands of 85, 99 and 106 nt were found at shorter incubation times. A fourth band (130 nt) was detected most clearly after 20 min of incubation (Figure 2C). These bands position the initiation points downstream of the CS-6 sequence, and in the vicinity of the RS_B (Figure 1B). Preliminary experiments in which the *E.coli* single-stranded DNA binding protein (SSB protein) was added to the pneumococcal extracts indicated that the replication
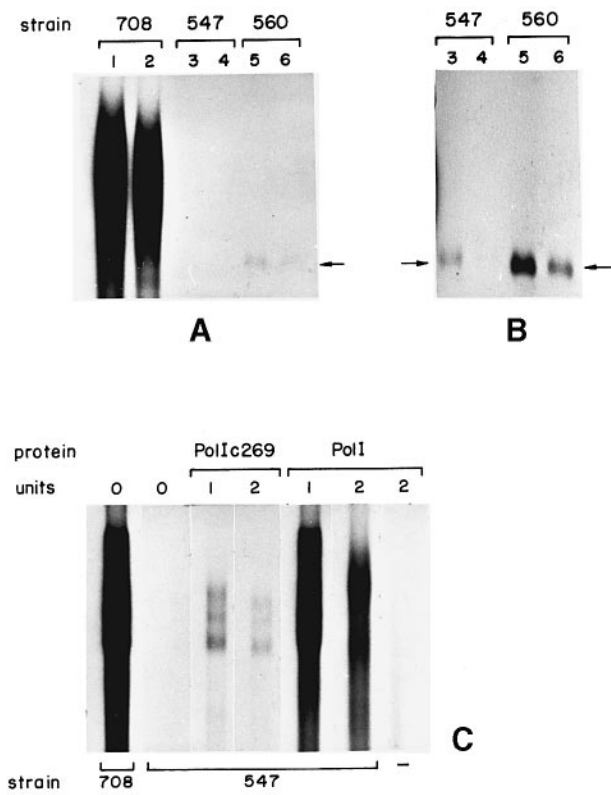
Fig. 3. Effect of Spn PolI on the *in vitro* replication of ssDNA. (**A**) Extracts prepared from pneumococcal strains, wild-type (708) or *polA*-deficient (547 and 560) cultures received ssDNA containing the pLS1-*ssoA* in the functional orientation. Proteins from the extracts were 240 µg (lanes 1, 3 and 5) or 480 µg (lanes 2, 4 and 6). Reaction products were run on 1% agarose gels without linearization. (**B**) An overexposed autoradiogram (lanes 3–6 from A) showed some replication products (arrows). (**C**) Complementation of *in vitro* replication of ssDNA by Spn PolI purified protein. Extracts from the indicated strains (708 and 547) received 0, 1 or 2 U of purified Spn PolI or Spn PolIc269 proteins. As a control, ssDNA and 2 U of purified Spn PolI, lacking pneumococcal extracts (–), were tested.

Fig. 4. Predicted secondary structures at the CS-6 region (upper part) and at the RS_B (lower part) of the pLS1-*ssoA* wild-type and the mutants constructed in this work. Coordinates in the wild type pLS1 DNA are indicated. Base changes are in bold letters. Only the regions affected by the mutations are shown.

start sites did not change, although there was an increase in the relative amounts of specific DNA replication (not shown). Treatment of the samples with alkali or with RNase A did not change the pattern of the bands (not shown), as previously observed for other RCR plasmids (Dempsey *et al.*, 1995). This finding indicates that the bands correspond to newly synthesized DNA fragments lacking any RNA. The size of these fragments would define the DNA–RNA transition points, if a pRNA is assumed to be synthesized in these extracts.

Spn PolI is involved in the initiation of lagging strand synthesis

Plasmids with the pLS1 replicon were shown to contain DNA discontinuities when tested in pneumococcal strains deficient in DNA polymerase I (Díaz *et al.*, 1994). One of these discontinuities was mapped in the vicinity of the pLS1-*ssoA*, and could correspond to molecules in which the ss→dsDNA conversion was not completed. In addition, the amount of ssDNA intermediates observed *in vivo* was higher in the Spn PolI-deficient strains than in the wild-type strain. These results indicated that Spn PolI plays a role in initiation and/or completion of lagging strand synthesis (Díaz *et al.*, 1994). To test directly whether the
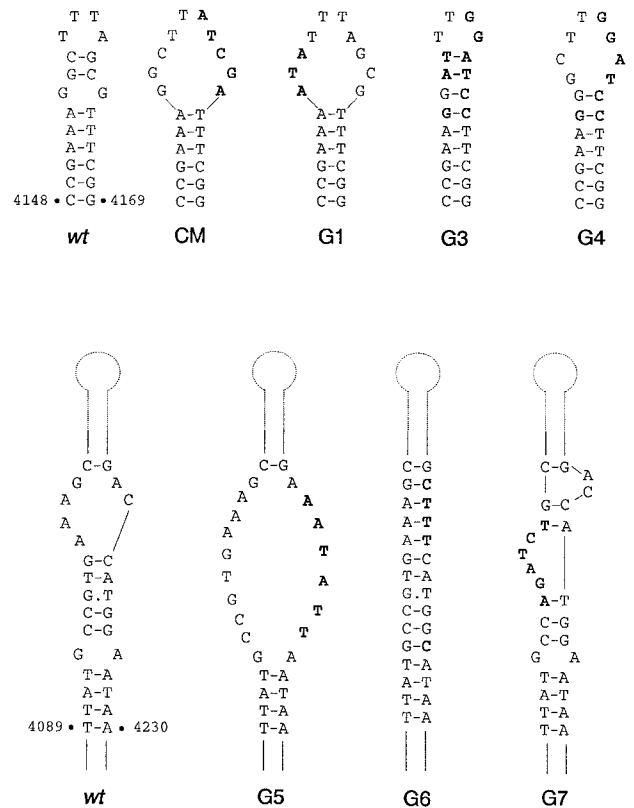
Spn PolI influenced initiation of lagging strand synthesis, cell-free extracts were prepared from two mutant strains, one lacking the polymerizing activity and having a reduced 5′–3′ exonuclease activity (~15% of the wild-type) (strain 547, synthesizing the PolIn351a polypeptide; P.López, personal communication), and the second having a reduced polymerization activity (to 20% of the wild-type) but with normal levels of 5′–3′ exonuclease activity (strain 560, synthesizing the polypeptides PolIc269 and PolIn351b; Díaz *et al.*, 1992). A strain fully defective in both activities is not available because the 5′–3′ exonuclease activity, unlike that of the *E.coli* DNA PolI, is essential for viability of *S.pneumoniae* (Díaz *et al.*, 1992). It is worth pointing out that the Spn PolI lacks the 3′–5′ exonucleolytic activity, and that the Spn PolIc269 is equivalent to the *E.coli* Klenow enzyme (Pons *et al.*, 1991; Díaz *et al.*, 1992). *In vitro* lagging strand synthesis was assayed in extracts from the wild-type strain (strain 708) and from both *polA*-deficient strains, using as a template ssDNA containing the pLS1-*ssoA*. The results (Figure 3A) showed that replication was drastically reduced in both extracts, the presence of partially replicated molecules being visible only after long exposure of the gels (Figure 3B). Lack of DNA synthesis was much more evident when the extracts were prepared from strain 547 (deficient in the polymerase activity). To verify whether these replicative defects could be corrected, extracts from this strain received ssDNA and purified Spn PolI or Spn PolIc269 proteins. The activity of the protein added corresponded roughly to the

Table II. Oligonucleotides and mutations introduced into the *ssoA* region of pLS1

Mutation	Oligonucleotide (5′–3′)[a]	New restriction site
G1	CGTGCCGAGCCGAAA**ATATT**TAGCGTTTCGGAC	*Ssp*I
G3	CGTGCCGAGCCGAAGG**ATTTGGATCC**TTCGGACGGACACGG	*Bam*HI
G4	CCGAGCCGAAGGGCTT**GGATCC**TTCGGACGGACACGG	*Bam*HI
G5	CTTGTTGTCAAATAGA**AATATT**AATAAAAAGCGTCAAAAGTCTTG	*Ssp*I
G6	CTTGTTGTCAAATAG**CTTT**CATGG**C**ATAAA**AAGCTT**CAAAAGTCTTGAGTGGA	*Hin*dIII
G7	TGAGCGATTTATGCC**AGATCT**GCTATTTGACAATAA	*Bgl*II
CM	CGAGCCGAAAGGCTT**ATCGA**TTTCGGACGGACACGGA	*Cla*I

[a]Changes introduced into the wild-type sequence are underlined; recognition sites for the indicated enzymes are marked in bold.

physiological concentration of Spn PolI (1 U) or twice this amount (López *et al.*, 1989; see Materials and methods). The results showed that only the complete Spn PolI was able to restore replication from the pLS1-*ssoA* DNA almost to wild-type levels (Figure 3C). From these results, we conclude that: (i) Spn PolI is involved in the *in vitro* initiation of lagging strand replication from the pLS1-*ssoA*; and (ii) both the polymerizing and the 5′–3′ exonuclease domains are required for lagging strand synthesis, the polymerase domain alone (Spn PolIc269) being much less efficient. In addition, since Spn PolI by itself, in the absence of any extract, failed to replicate ssDNA (Figure 3C), host factor(s) other than Spn PolI are required to initiate DNA synthesis (perhaps RNAP, see below).

Construction of mutants in the ssoA
We considered the RS$_B$ and the CS-6 conserved sequences as appropriate targets to construct pLS1-*ssoA* mutants because: (i) *sso* functionality depends upon its orientation both *in vivo* (Gruss *et al.*, 1987; Kramer *et al.*, 1995) and *in vitro* (Dempsey *et al.*, 1995; Figure 2B), and thus unpaired regions are likely to be important for *sso* activity; (ii) pneumococcal cells harbouring plasmids bearing deletions that remove the RS$_B$, but not the CS-6, accumulate less ssDNA than plasmids carrying a total *ssoA* deletion (Kramer *et al.*, 1995); and (iii) a plasmid mutant with slight changes at the CS-6 (CM mutation; Figure 4) accumulates twice as much ssDNA as the wild-type plasmid in *S.pneumoniae* cells (Kramer *et al.*, 1995). To perform the mutagenesis, ssDNA from the recombinant pApLS1ssoA⁻ was subjected to site-directed mutagenesis with the desired oligonucleotides (Table II). Employment of the phagemid and tagging the mutations with a recognition site for a restriction enzyme (Table II) provided us with an easy procedure to construct and select for the desired mutations with a high frequency. The mutations (Figure 4) were designed to introduce small changes in the region encompassing the CS-6 (CM, G1, G3 and G4 mutants), the RS$_B$ (G5–G7) or both (G3G7 double mutant). Changes in the local *ssoA* structure without changes in the conserved sequences (mutants G1, G5 and G6) were designed to vary only the intrastrand pairings (Figure 4). We failed in constructing plasmids that contain a complete pairing within the CS-6, perhaps because of physical constraints on the DNA, which would support the prediction that the CS-6 is located on the terminal loop of the *ssoA* hairpin (del Solar *et al.*, 1987a). These changes did not alter the high potential for secondary structure formation within the *ssoA*, and only local changes in the

general organization of this region were predicted by computer analysis (not shown). This set of mutants constitute the first attempt at performing a fine analysis of the structure and function of the lagging strand origins of RCR plasmids. In addition, since they have small, albeit critical, changes within the *ssoA*, we could use these to assess the role of both the conserved sequences (RS$_B$ and CS-6) in lagging strand replication.

Binding of RNAP to ssDNA containing the pLS1-ssoA
Streptococcus pneumoniae cells harbouring pLS1-based plasmids bearing mutations in the RS$_B$ region (G5–G7) accumulated almost the same amount of intracellular ssDNA as plasmids lacking the *ssoA*. However, the amount of ssDNA accumulated by plasmids with mutations in the CS-6 increased only slightly compared with the wild-type (Kramer *et al.*, 1995; our unpublished observations). One possible explanation for this would be that the RS$_B$ is involved either directly or indirectly in interaction with a host factor. Since one candidate could be RNAP, we decided to test whether this protein specifically binds to ssDNA harbouring the *ssoA*. To this end, we used purified RNAP from *B.subtilis* because the enzyme originates from a Gram-positive bacterium like *S.pneumoniae*. As target DNAs we synthesized ssDNA fragments (313 nt) by asymmetric PCR carrying the pLS1-*ssoA* sequence, its complementary (ssoA⁻), and those harbouring the aforementioned mutations. Since the functionality of the *ssoA* is orientation dependent (Gruss *et al.*, 1987), the target DNAs would contain the *ssoA* in the functional or non-functional orientation. Formation of complexes between RNAP and labelled ssDNA (3 ng per reaction) was tested by gel retardation assays in the presence of an excess of competitor [poly(dI–dC), 1 mg per reaction]. The results showed that RNAP–ssDNA complexes were generated readily when the target DNA contained the pLS1-*ssoA* in the functional orientation (Figure 5A). At the highest RNAP/ssDNA molar ratio tested (3.4), >90% of the input ssDNA was complexed with RNAP. At the same ratio, only 20% of the ssDNA was complexed with the protein when the ssDNA used contained the pLS1-*ssoA* in the non-functional orientation (Figure 5A). These results showed that RNAP binds specifically to ssDNA containing the pLS1-*ssoA*.

We next performed similar assays, but employing ssDNA with mutations in the CS-6 and RS$_B$ regions. The results (Figure 5B and C) can be summarized as follows: changes in the CS-6 did not affect the ability of RNAP to bind to ssDNA (Figure 5B), whereas all changes in the
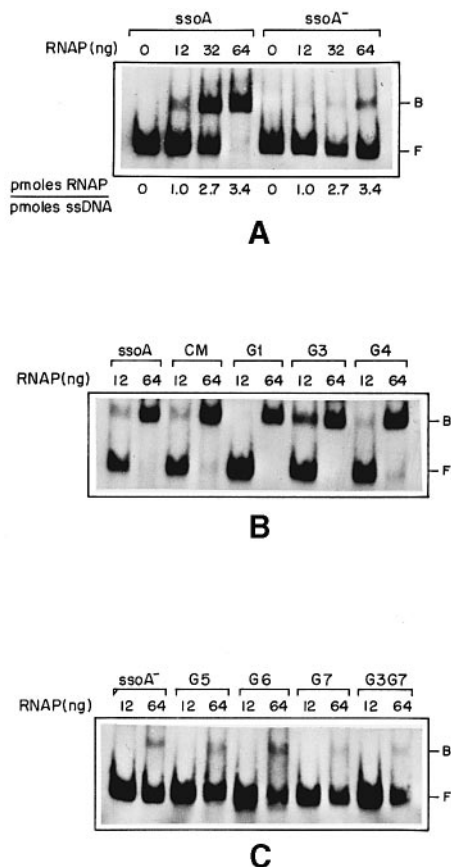
Fig. 5. Gel retardation assays of RNAP bound to labelled ssDNA fragments. (**A**) ssDNAs harbouring the pLS1-*ssoA* in the functional (ssoA) or in the non-functional (ssoA⁻) orientation were incubated with the indicated amounts of RNAP (in pmol) at the ratios indicated. (**B**) and (**C**) Similar experiments performed with ssDNA substrates prepared from templates harbouring mutations in the CS-6 sequence (B), in the RS$_B$ or in both (C) of the pLS1-*ssoA*. Positions of the bound (B) and free (F) ssDNAs are indicated.

RS$_B$ behaved like the ssDNA containing the pLS1-*ssoA* in the non-functional orientation (Figure 5C). We conclude that either the sequence or the strucural elements in the RS$_B$ are important for the binding of the RNAP to the pLS1-*ssoA*, and that the CS-6 is not involved in this binding.

RNAP-mediated synthesis of a pRNA from the pLS1-ssoA

Since RNAP binding to the pLS1-*ssoA* was efficient and specific, we tested whether the enzyme was able to synthesize (a) primer RNA(s) using ssDNA as template. Based on the above results, we hypothesized that pRNA synthesis should not be feasible when the template DNA has the *ssoA* in the non-functional orientation, or when it harbours mutations in the RS$_B$. The reaction mixtures contained unlabelled ssDNA fragment (0.1 pmol) and RNAP (1 pmol) and were performed either in the presence or absence of the *E.coli* SSB protein (28.5 pmol). The amount of SSB protein was calculated to be in excess, to coat all the ssDNA used. Reactions were started by the addition of [α-³²P]UTP in the presence of unlabelled ribonucleotides. Heparin (5 mg per reaction) was added to the mixtures to ensure a single round of transcription. Analysis of the products obtained with the wild-type *ssoA*-
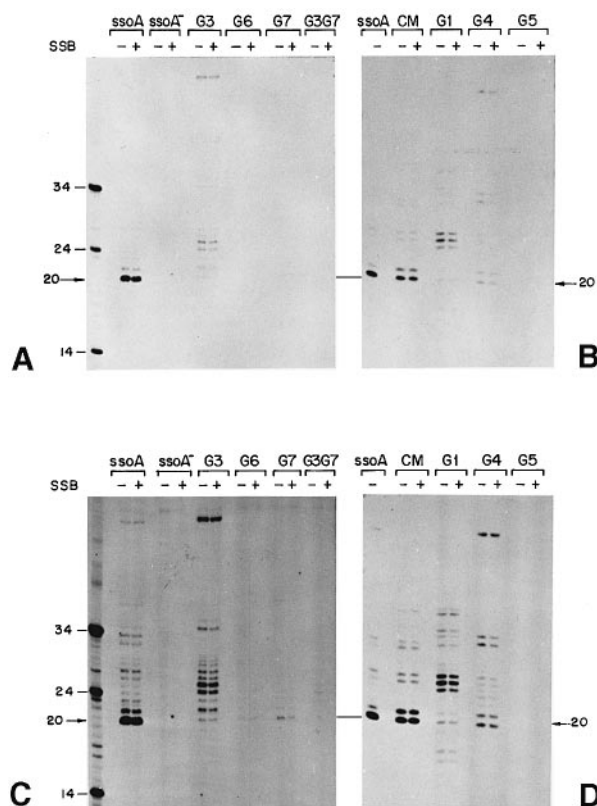

Fig. 6. RNAP-directed synthesis of pRNAs from various ssDNAs. The ssDNAs were prepared by asymmetric PCR from templates harbouring the wild-type pLS1-*ssoA* sequence or the indicated mutations. Samples were incubated in the absence (–) or presence (+) of the *E.coli* SSB protein. The position of DNA markers (leftmost lanes) are indicated. The size of the major pRNA product from the wild-type template (20 nt) is indicated. (**C**) and (**D**) Overexposed autoradiograms of the pRNA bands shown in (A) and (B).

containing ssDNA fragment (Figure 6) showed that RNAP synthesized a pRNA with a size of 20 nt. A minor species (21 nt, 10% of the main band) was also detected in short exposures of the gels (Figure 6A and B). When the gels were exposed for longer times (Figure 6C and D), a ladder of bands, ranging from the 20 nt main product up to 30 nt, was detected. These minor products amounted to <5% of the total RNA synthesized. No trace of pRNA was found when the template DNA contained the *ssoA* in the non-functional orientation (ssoA⁻). In this case, only a few very faint bands corresponding to high molecular weight species were visible in overexposed gels (Figure 6C). No differences were found when the SSB protein was added to the reaction mixtures, even though the ssDNAs used were able to bind the protein with a similar efficiency (not shown). We conclude that, *in vitro*, the *B.subtilis* RNAP is able to synthesize a short pRNA from the pLS1-*ssoA* in the absence of other host factors.

When the ssDNA template contained slight changes in the CS-6 sequence (mutant CM, Figure 6B and D), the pRNA species synthesized corresponded mainly to that obtained with the wild-type *ssoA*. Although a slight increase in the amount of the longer species synthesized was evident, the level of RNA synthesis of the major product was only reduced to 70% of the wild-type. In the case of the other mutations in the terminal loop (G1–G4), the proportion of longer pRNA species increased, and the
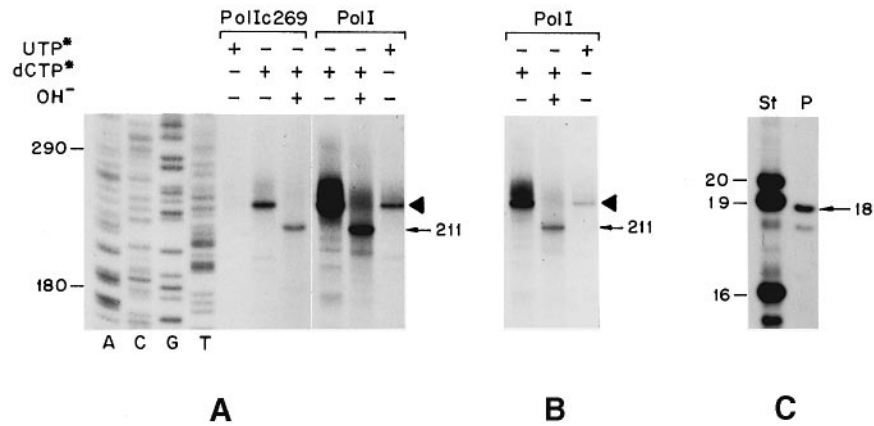
Fig. 7. (**A**) Determination of the 3′ end of the pRNA and DNA synthesis from the pRNA-primed ssDNA substrate by the Spn PolI and the Klenow-like Spn PolIc269 polymerases. The newly synthesized DNA was labelled with [α-^{32}P]dCTP (*) or not labelled, but in this case the pRNA was labelled with [α-^{32}P]UTP (*) prior to incubation with the DNA polymerases. In both cases, a labelled band of 230 nt was visible (◄). To determine the 3′ end of the pRNA, reactions were incubated with alkali (OH⁻), which revealed a reduction in the size of the labelled product to 211 nt. A lighter exposure of the autoradiogram of samples treated with Spn PolI is shown in (**B**). ACGT, sequencing ladder generated by the Sanger method (Sambrook *et al.*, 1989). Numbers indicate sizes in nucleotides. (**C**) The 5′ end of the pRNA was identified by primer extension with AMV reverse transcriptase using a 13mer oligonucleotide. A major 18 nt and a minor 17 nt product (P) were observed. Their sizes were corrected for the presence of a phosphate (Sambrook *et al.*, 1989) in the mixture of labelled oligonucleotides that were used as standards (St).

RNA synthesis level was reduced to 50%. These results indicate that the mutations at CS-6 do not have a strong influence on the priming efficiency. In the case of ssDNA harbouring changes in the RS$_B$ region (G5–G7 and G3G7 double mutant), no significant synthesis of pRNA was observed, perhaps with the exception of the G7 single mutant in which a faint band of 20 nt (representing ~1% of the RNA synthesis level of the wild-type) was detected in overexposed gels (Figure 6C). From this set of results, we draw the following conclusions: (i) the sequence in the terminal loop encompassing the CS-6 seems to function as a transcriptional terminator for RNAP-directed pRNA synthesis; and (ii) changes in the RS$_B$ abolished, or drastically reduced, pRNA synthesis.

Mapping the 5′ and 3′ ends of pRNA synthesized from the wild-type ssoA

To identify the termination point of the pRNA and, at the same time, to link the pRNA synthesis with the polymerization stage of lagging strand synthesis by DNA PolI, ssDNA (containing the wild-type *ssoA*) was primed with the pRNA by using the *B.subtilis* RNAP. This pRNA-primed ssDNA substrate was treated with 2 U of the Spn PolI or the Spn PolIc269 (Klenow-like) enzymes in the presence of [α-^{32}P]dCTP, and the products were analysed (Figure 7). To ensure that DNA synthesis was the result of the elongation of pRNA-primed ssDNA molecules, controls of the same reaction were carried out in which the pRNA had been labelled with [α-^{32}P]UTP and the newly synthesized DNA was unlabelled. In all reactions, synthesis of a major 230 nt DNA species was observed (Figure 7A and B). This size corresponds to a run-off synthesis of DNA which would start from a pRNA of 20 nt having its 3′ end around the CS-6 region. The precise position of the 3′ end of the pRNA was determined by treatment of the reaction mixtures with alkali (to remove the RNA species), which reduced the size of the bands to 211 nt (Figure 7A and B). This positioned the 3′ end of the pRNA at the pLS1 coordinate 4156, which agrees with the 130 nt band obtained in experiments to

map the initiation site of DNA synthesis (Figure 2C; see Figure 8A). When the polymerizing domain (Spn PolIc269) was used instead of the entire Spn PolI enzyme, the efficiency of DNA synthesis was reduced 10-fold (Figure 7A), which confirms the requirement for the 5′–3′ exonuclease domain of Spn PolI to achieve a high rate of DNA polymerization. Other DNA polymerases tested (*Taq*, and from phages T4 and T7) exhibited very low efficiency of synthesis (not shown). We conclude that the pneumococcal Spn PolI is able to elongate the pRNA synthesized by the RNAP, and that the pRNA terminates at coordinate 4156.

To determine the 5′ end of the pRNA, an assay of primer extension of ssDNA primed with the pRNA was developed. A 13mer oligonucleotide (5′-TTAGCGTTT-CGGA-3′, corresponding to coordinates 4158–4170; Figure 1B), which should hybridize with the pRNA, was annealed to the primed substrate. The mixture was elongated with avian myeloblastosis virus (AMV) reverse transcriptase, and the labelled products were resolved on sequencing gels. A major product of 18 nt was synthesized (Figure 7C), which would position the 5′ end of the pRNA at coordinate 4175, indicating that the first ribonucleotide incorporated into the pRNA would be a G (see Figure 8A). A 17 nt minor product (representing ~20% of the total) was observed, which would either correspond to a premature release of the reverse transcriptase from its template, or indicate the existence of a minor pRNA species initiating at coordinate 4174. Since this latter product would point to a pRNA initiated by a T residue, we consider this second possibility unlikely. Taking the above results together, we conclude that the *in vitro* synthesized pRNA of the pLS1-*ssoA* is 20 nt long, spanning from coordinates 4175 to 4156 (Figure 8A).

Discussion

We have defined here two proteins which are essential for *in vitro* initiation of lagging strand replication from the *ssoA*: RNAP and DNA PolI. Whereas the former is
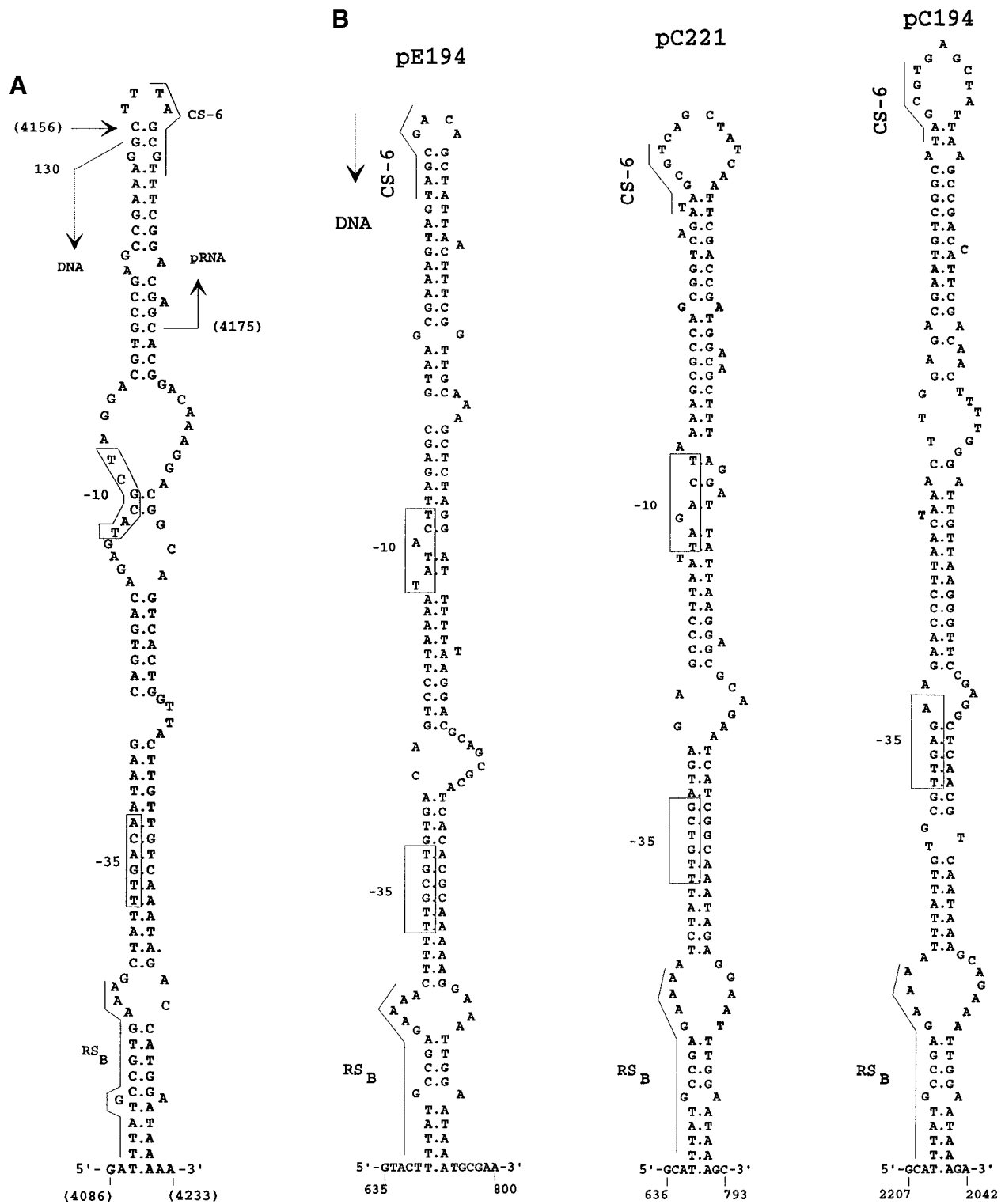
A

B

pE194

pC221

pC194

Fig. 8. Possible promoter-like sequences on the *ssoA* region of some RC plasmids. (**A**) Proposed structure of the pLS1-*ssoA* between coordinates 4086 and 4233. The positions of the RS$_B$ and the CS-6 sequences are indicated. Regions −35 and −10 of the putative promoter-like sequences are boxed. The initiation and termination sites of the pRNA (from coordinates 4175 to 4156) are indicated by solid arrows. The transition point from RNA to DNA synthesis, which would yield a 130 nt band observed in the pneumococcal extracts (Figure 2), is also indicated. (**B**) Proposed −35 and −10 regions in the *ssoA* of some staphylococcal plasmids sharing similarities with the pLS1-*ssoA*. The direction of DNA synthesis in the pE194-*ssoA* (Dempsey *et al.*, 1995) is indicated.

required for the synthesis of a 20 nt long pRNA, DNA PolI is needed for extension of the pRNA. In addition, we have clarified the role of two conserved sequences present in the *ssoA*-type lagging strand origins.

Although it was postulated that RNAP should be the main host factor involved in the initiation of plasmid lagging strand replication, no direct evidence for the existence of a pRNA has been described so far (Dempsey

et al., 1995). This contrasts with the filamentous ssDNA coliphages, in which the existence of a pRNA synthesized at a single site on the viral DNA by the *E.coli* RNAP has been demonstrated earlier (Geider and Kornberg, 1974; see Kornberg and Baker, 1992). In the case of RCR plasmids, the role of regions involved in lagging strand replication has been investigated either by *in vivo* analysis of plasmid derivatives with partial or total deletions of the *ssoA* (del Solar *et al.*, 1987a; Kramer *et al.*, 1995) or by *in vitro* determination of the start site of DNA synthesis (Dempsey *et al.*, 1995). The former approach, however, gives an all-or-none response because only the amount of intracellular ssDNA accumulated can be measured. The mutations constructed here in the conserved unpaired regions within the pLS1-*ssoA* have revealed their importance for lagging strand synthesis. In addition to their importance in the *in vitro* assays, both conserved sequences play a role *in vivo* as signals for the ss→dsDNA conversion (our unpublished results). Since pRNA transcription initiates at coordinate 4175, an inspection of the predicted intrastrand pairings within the pLS1-*ssoA* revealed the existence of a putative promoter-like structure in the vicinity of the RS_B (Figure 8A). Such a putative promoter would have a consensus –35 region (5′-TTGACA-3′) but a weak –10 region (5′-TAcgcT-3′). A similar promoter-like region for RNAP-dependent primer synthesis was observed in filamentous coliphages when the sequence of the pRNA was determined (Higashitani *et al.*, 1993), and single-stranded promoter regions are found in the coliphage N4 (Gluksmann-Kui *et al.*, 1996). The organization of this kind of promoters is such that they are located on the DNA strand that is partially complementary to the template strand. Thus, RNA synthesis should start and proceed in the direction toward the binding site of RNAP, in a situation that is opposite to RNA synthesis from classic promoters.

The *ssoA*s of various RCR plasmids with conserved RS_B and CS-6 regions have a structure which is similar to that of the pLS1-*ssoA* (del Solar *et al.*, 1987a; Gruss *et al.*, 1987; Dempsey *et al.*, 1995). In addition, promoter-like sequences are found in the vicinity of the RS_B, resembling the pLS1-*ssoA* (Figure 8B). If these sequences also act as promoters for pRNA synthesis, the DNA initiation points found for *ssoA*-containing RCR plasmids (Dempsey *et al.*, 1995; Figure 8B) would fit within a general picture in which the mechanism for ss→ds DNA conversion would be similar to that of pLS1.

The pRNA ends at coordinate 4156, just after the terminal loop of the predicted *ssoA* secondary structure, which would act as a transcriptional terminator (Wilson and von Hippel, 1995, and references therein). Alterations in the sequence or structure of the CS-6 lead to: (i) normal binding of the RNAP to its target (Figure 5B); and (ii) failure of RNAP to terminate at this position, thus generating pRNAs of longer sizes (Figure 6). Transition of pRNA to DNA around the CS-6 region would account for the 130 nt long band observed in partially replicated DNA molecules in the pneumococcal extracts (Figure 2C). This band would correspond to the distance from the cleavage site of *Afl*II to the G at position 4157, from which Spn PolI would extend the pRNA (Figure 7). The smaller bands (85, 99 and 106 nt) observed in the pneumococcal extracts could be explained by synthesis of

longer (or different) pRNA species by the *S.pneumoniae* RNAP. However, since similar results have been found for other *ssoA*-containing plasmids in *S.aureus* extracts (Dempsey *et al.*, 1995), this possibility seems unlikely. Alternatively, the 5′–3′ exonuclease of the Spn PolI after degradation of the pRNA could continue further on the newly synthesized DNA and could stop at preferential points (see below). At the present stage of knowledge, all of these interpretations are still speculative.

Our model for pRNA synthesis postulates that RNAP recognizes the RS_B region as the primary binding/positioning site within the *ssoA*. Although the mutations introduced in the RS_B do not directly affect the putative promoter, they result in the alteration of the –40 to –50 upstream regions, which are important for promoter activity (Buckle *et al.*, 1991). If the RS_B region acts as a UP enhacer element (Ross *et al.*, 1993), these mutations would lead to the observed failure in the RNAP binding and, as a consequence, in the lack of pRNA synthesis. Since the *B.subtilis* RNAP used was able to perform synthesis of the pRNA, we believe that the lack of functionality of the pLS1-*ssoA* in this host (del Solar *et al.*, 1993b; Kramer *et al.*, 1995; Meijer *et al.*, 1995b) is due to the inefficient activity of other host factor(s) required for the initiation of lagging strand synthesis (DNA PolI?) rather than due to a lack of recognition of the *ssoA* by the RNAP. Since we have no information on the pneumococcal RNAP, we cannot rule out the possibility that these effects can be due to differences in the affinity of the *B.subtilis* RNAP and its pneumococcal counterpart for the pLS1-*ssoA*.

The role of DNA PolI in ssDNA replication is more difficult to assess. Two lines of evidence suggest an essential role for the enzyme in RC plasmid replication. Firstly, the RCR plasmid pEP2 was shown to be lost in an *E.coli* strain carrying a temperature-sensitive allele of *polA* when cultures were grown at the non-permissive temperature (Zhang *et al.*, 1994). Secondly, the observed *in vivo* effects on pLS1 replication, namely the increase in the intracellular amount of ssDNA and of discontinuities in the dsDNA (Díaz *et al.*, 1994). Our results obtained with the pneumococcal extracts showed that strains defective in the Spn PolI failed to support lagging strand synthesis. This defect could be corrected by the addition to the extracts of the Spn PolI protein, but not of the Spn PolIc269 polypeptide, which agrees with the ability of the Spn PolI enzyme (but not of Spn PolIc269) to elongate the pRNA efficiently. Consequently, the entire Spn PolI is essential for replication from the *ssoA*, and uncoupling of the two domains of the enzyme (polymerizing and 5′–3′ exonucleolytic activities) could abolish its activity for lagging strand synthesis. In the case of plasmid ColE1, *in vitro* elongation of the RNA primer is performed only by the *E.coli* DNA PolI, but not by the Klenow fragment (Itoh and Tomizawa, 1978). One explanation for the lack of pRNA attached to the newly replicated products in the *in vitro* replication systems (Dempsey *et al.*, 1995; this work) is that more than one molecule of Spn PolI participates in the process. We could envisage that one molecule of the DNA PolI would elongate the pRNA for some 80–100 nt, whereas another molecule would remove the pRNA. Alternatively, we could speculate on the existence of a 'threading' mechanism, such as the one

reported for the bacteriophage T5 5′-exonuclease (Ceska *et al.*, 1996), in which the pRNA could slide through a helical arch in the exonucleolytic domain of the Spn PolI, followed by its degradation. Any of these possibilities could explain the failure to observe pRNAs in the staphylococcal (Dempsey *et al.*, 1995) and pneumococcal extracts because, at the time of assay, pRNA would already be removed. However, degradation of the pRNA by the Spn PolI was not observed when the protein was added to pRNA-primed ssDNA, perhaps because the divalent cation used in this experiment (Mg^{2+} instead of Mn^{2+}) favoured polymerization over degradation, and the amount of dNTPs used was enough to reduce the 5′–3′ exonuclease activity of the pneumococcal enzyme (P.López, personal communication). At a later stage, the bulkier DNA PolIII would continue lagging strand synthesis.

The efficiency with which the *sso* is recognized by the host machinery is important for the ability of plasmids to colonize new hosts (del Solar *et al.*, 1996). Since the efficiency of *ssoA* recognition (measured by intracellular accumulation of ssDNA) seems to be host-specific, perhaps host factors other than, or in addition to, the host RNAP and DNA PolI participate in the recognition of the *ssoA* for lagging strand replication. It is assumed that the ssDNA plasmid intermediates are covered by the host SSB protein, like the ssDNA coliphages, although (in our experimental conditions) we have not found a major influence of the *E.coli* SSB protein in the RNAP-directed synthesis of pRNA. Nevertheless, it is possible that, *in vivo*, binding of a host-specific SSB-like protein to the ssDNA plasmid intermediates would facilitate generation of secondary structures by intrastrand pairing of the inverted repeats located within the *sso*, as shown for the ssDNA coliphage G4 (Sun and Godson, 1993) and for phage N4 (Glucksmann-Kuis *et al.*, 1996). Hairpins at the *sso* would facilitate the generation of a promoter-like structure in the vicinity of the RS_B. Other host factors could bind to the *ssoA* region, generating a tertiary structure that could facilitate RNAP binding. In this sense, homology between the RS_B and the gyrase-binding site present in plasmid pSC101 (Wahle and Kornberg, 1988) has been reported, although its significance is not clear at present (Novick *et al.*, 1984; del Solar *et al.*, 1993b).

Materials and methods

Bacterial strains and plasmids
Strains and plasmids employed are listed in Table I. Media and growth conditions have been described (Lacks *et al.*, 1986; Sambrook *et al.*, 1989). To construct the recombinant phagemids, either the 1134 bp *Hin*dIII–*Pst*I or the 1243 bp *Eco*RI–*Pst*I fragment of pLS1 was cloned into phagemid pALTER-1 digested with the same enzymes (Table I). These constructions place the pLS1-*ssoA* in the functional (pApLS1ssoA) or in the non-functional (pApLS1ssoA⁻) orientation. *Escherichia coli* cultures harbouring the aforementioned phagemids were selected for resistance to tetracycline (15 µg/ml).

Site-directed mutagenesis
An Altered Sites kit (Promega), designed for *in vitro* mutagenesis, was used. To perform the mutagenesis, the 1243 bp *Eco*RI–*Pst*I fragment of pLS1 (coordinates 3170 and 5, respectively; Lacks *et al.*, 1986) was cloned into the pALTER-1 vector digested with the same enzymes. Mutagenesis was performed following the protocol from Promega, and using the oligonucleotides listed in Table II. The mutations generated the indicated restriction sites to facilitate the screening. Phagemids harbouring the desired mutations were isolated from *E.coli*, and the

mutations were identified by digestion with the appropriate restriction enzymes. The cloned fragments were inserted back into pLS1 by swapping the *Eco*RI–*Pst*I fragments. The resulting plasmids were rescued by transformation of *S.pneumoniae*, and the mutations were characterized by determining the entire nucleotide sequence of the *ssoA*, using the T7 Sequencing kit (Pharmacia). These DNAs were used as templates for asymmetric PCR (see below).

Isolation and preparation of ssDNA
To generate ssDNA for the *in vitro* replication assays with pneumococcal extracts, *E.coli* JM109 cells containing the recombinant phagemids were infected at a multiplicity of infection of 10 with the RK408 helper f1-derived bacteriophage. Cultures were grown for 13–16 h at 37°C, and the encapsidated ssDNA was precipitated and purified as described (Sambrook *et al.*, 1989). DNA products were analysed by agarose gel electrophoresis and the concentration of ssDNA was determined by densitometric scanning.

Generation of ssDNA for the gel retardation assays and for the *in vitro* transcription with RNAP was performed by asymmetric PCR using as template 10 ng of pLS1 DNA or of its derivatives containing the mutations in the *ssoA*. The oligonucleotides used were: UP (5′-TCA-GCAAAATGACAAGATGCTAGG-3′; 50 pmol) and LO (5′-TCAT-CCACTCAAGACTTTTGAGCA-3′; 0.5 pmol). When the ssDNA to be amplified was complementary to the pLS1-*ssoA*, the concentrations of oligos UP and LO were reversed. The PCR kit from Boehringer Mannheim was used, and the reactions were performed in the presence of 5 U of *Taq* DNA polymerase. DNA amplification was achieved by 30 cycles (60 s at 94°C, 60 s at 48°C and 90 s at 72°C), followed by two more cycles (60 s at 94°C, 60 s at 48°C and 300 s at 72°C). DNA was extracted from the reaction mixtures and electrophoresed on a preparative 2% agarose gel. The ssDNA was purified with the aid of the Mermaid kit (Bio101). Labelling of ssDNA samples at their 5′ ends was performed with [γ-^{32}P]dATP and polynucleotide kinase as described (Sambrook *et al.*, 1989).

Preparation of cell-free extracts
Streptococcus pneumoniae extracts were prepared essentially as described for *S.aureus* (Birch and Khan, 1992), except that the cultures were harvested at ~2×10^8 colony-forming units per ml of culture and lysis was performed in buffer containing 50 mM sodium phosphate buffer (pH 6.9), 5 mM EGTA, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and pneumococcal autolysin (10 µg/ml). Lysis was completed by incubation at 37°C for 7 min. Yield of proteins was ~55 mg/ml. The extracts were divided into 25 µl aliquots and kept at –70°C.

In vitro replication assays
The assays were performed as described (Birch and Khan, 1992). Reaction mixtures (30 µl) contained 150 ng of ssDNA, 240 µg of bacterial proteins, incubation buffer [40 mM Tris, pH 8.0, 100 mM KCl, 12 mM Mg(OAc)$_2$, 1 mM dithiothreitol (DTT)], ‘energy mixture’ (50 µM NAD, 50 µM cAMP, 2 mM ATP), nucleotides (0.5 mM each of UTP, CTP and GTP, 50 µM each of dCTP, dGTP and dTTP) and 20 µM [α-^{32}P]dCTP (3000 Ci/mmol). Details of the optimization of the pneumococcal replication system will be published elsewhere (M.G.Kramer, S.A.Khan and M.Espinosa, in preparation). When the replication extracts were supplemented with purified Spn PolI protein (M_r 99.5 kDa, specific activity of the preparation 0.38 U/µg), the calculated amount of 500 molecules of protein per cell was used, to resemble the physiological amount of enzyme (López *et al.*, 1989). This would correspond to 1 U of enzyme per assay. Incubations were carried out at 32°C at various times, and the replication products were extracted with phenol–chloroform and precipitated with ethanol. Reaction products were separated by electrophoresis on 1% agarose gels in the presence of ethidium bromide (0.5 µg/ml), or in 8% acrylamide sequencing gels. To convert the various forms of DNA to a single band, DNAs were digested with *Hin*dIII and electrophoresed on 1% agarose gels as above. Bands were visualized under UV irradiation and by autoradiography of the dried gels. The positions of the replicated products were identified using DNA markers on the same gel.

Determination of the initiation sites of lagging strand replication
The procedure described earlier (Dempsey *et al.*, 1995) was followed. Replication was performed as above, using as templates ssDNAs (150 ng) isolated from the phagemid pApLS1ssoA or from pALTER-1. DNA present in the replication products was recovered by phenol extraction and ethanol precipitation. One-half of the DNA samples were digested

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with *Afl*II, which cuts downstream of the expected initiation sites of DNA replication. Samples were denatured by boiling, and digested and undigested samples were loaded on an 8% polyacrylamide–8 M urea sequencing gel. Similar results were found when 40% formamide was included in the gels. Known sequence reactions were run in the same gels as size markers.

Binding of RNAP to ssDNA

Reaction mixtures (10 µl) contained 3 ng (0.03 pmol) of 5′-end-labelled 313 nt long ssDNA (~30 000 c.p.m.) in binding buffer (25 mM Tris pH 7.5, 200 mM NaCl, 90 mM ammonium sulfate, 10 mM MgCl$_2$, 4% sucrose, 1 mM DTT), 1 µg of poly(dI–dC) and bovine serum albumin (BSA; 1 µg). *Bacillus subtilis* RNAP holoenzyme (pre-incubated with saturating amounts of sigma-A subunit) was prepared as described (Mencía *et al.*, 1996), and was added at the concentrations indicated in Results. Binding was performed by incubation at 4°C for 10 min, and samples received 2 µl of 30% glycerol prior to loading on 3.5% native polyacrylamide gels. Electrophoresis was performed at 4°C at 12.5 V/cm. The DNA–protein complexes were visualized by autoradiography of the gels and the bands were quantified by densitometric scanning of the autoradiograms.

Synthesis of pRNA

Bacillus subtilis RNAP-directed synthesis of pRNAs was performed in a total volume of 50 µl. ssDNA (313 nt; 0.1 pmol) in binding buffer was heated (90°C, 3 min), and slowly cooled to room temperature prior to the addition of 200 µM each of ATP, CTP and GTP, 80 µM UTP and [α-^{32}P]UTP (800 Ci/mmol). To ensure a single round of transcription, heparin (5 µg) was added to the mixtures. Synthesis was started by adding RNAP (1 pmol) and incubating at 37°C for 10 min. Reactions were stopped by adding 5 µl of a solution containing 100 mM EDTA and 2 µg/µl tRNA. Samples were ethanol precipitated and the labelled transcripts were analysed by electrophoresis on 15% polyacrylamide sequencing gels (Sambrook *et al.*, 1989). When the *E.coli* SSB protein was used, 28.5 pmol were added and it was incubated with the template ssDNAs at 30°C for 20 min before the addition of the nucleotides and RNAP. Bands were quantified as above.

In vitro elongation of the pRNA

From the 313 nt ssDNA template containing the wild-type *ssoA*, ^{32}P-labelled or unlabelled pRNAs were synthesized as above, with the exception of increasing cold UTP to 200 µM when pRNA was unlabelled. After phenol treatment and ethanol precipitation, samples were dissolved in 100 µl of reaction buffer (40 mM Tris pH 8.0, 6.5 mM MgCl$_2$, 5 mM DTT) containing 50 µg BSA and 150 µM dNTPs. When labelled DNA was synthesized, cold dCTP was omitted and 50 µM [α-^{32}P]dCTP (3000 Ci/mmol) was used. Purified Spn PolI or Spn PolIc269 enzymes (2 U) were added to these mixtures, and the reactions allowed to proceed for 30 min at 37°C. Polymerization was stopped by adding 40 mM EDTA, and the products were phenol treated and precipitated with ethanol. Some samples were treated with alkali (0.3 M NaOH, 1 mM EDTA) for 2 h at 37°C, followed by neutralization and ethanol precipitation. Samples were dissolved in loading buffer, and the reaction products were analysed by electrophoresis on 8% sequencing gels (Sambrook *et al.*, 1989) and autoradiography. Bands were quantified as above. Known sequence reactions were run in the same gels as size markers.

Primer extension

From the 313 nt ssDNA template containing the wild-type *ssoA* (0.5 pmol), unlabelled pRNA was synthesized as above, and samples were dissolved in water. The oligonucleotide 5′-TTAGCGTTTCGGA-3′ (1 pmol) was annealed to the substrate in a final volume of 14 µl in AMV reverse transcriptase buffer (50 mM Tris pH 8.3, 50 mM KCl, 10 mM MgCl$_2$, 1 mM DTT, 0.5 mM spermidine) containing 25 U of RNasin. Samples were heated to 65°C for 5 min and slowly cooled to room temperature. Then, 100 µM dATP, dGTP and dTTP, 10 µM [α-^{32}P]dCTP (3000 Ci/mmol) and 8 U of AMV reverse transcriptase (Promega) were added. Samples were incubated at 42°C for 30 min, and reaction products were recovered by phenol treatment and ethanol precipitation. Samples were dissolved in loading buffer and electrophoresed on 20% sequencing gels. As controls, labelled oligonucleotides of known sizes were also run. Gels were autoradiographed and quantified as above.

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