Lagging-strand origins of the promiscuous plasmid pMV158: physical and functional characterization

M. Gabriela Kramer, Gloria del Solar and Manuel Espinosa

Author for correspondence: Manuel Espinosa. Tel: +34 1 561 1800 (ext. 4209). Fax: +34 1 562 7518. e-mail: CIBMEl3@CC.CSIC.ES

The streptococcal plasmid pMV158 replicates by a rolling circle mechanism, which involves the generation of single-stranded plasmid DNA intermediates. This plasmid has the unique feature of having two lagging-strand origins of replication. One of these origins, termed ssoU, is functional in *Streptococcus pneumoniae* and in *Bacillus subtilis* in an orientation-dependent manner. The other origin, ssoA, is only functional in the former host. RNA polymerase seems to be involved in the initiation of the conversion of single- to double-stranded plasmid DNA from both ssoA and ssoU. Mutational and deletion analyses have allowed us to define ssoA as being within a highly structured, non-coding 199 bp region. Within this region, two elements which are conserved in several rolling-circle replicating plasmids are located, the recombination site RS, and a 6 base consensus sequence. Both elements may play a role in the conversion of single- to double-stranded plasmid DNA.

**Keywords:** Lagging-strand origins, RNA polymerase, rolling-circle replication, single-stranded DNA

---

**INTRODUCTION**

Circular, double-stranded (ds) bacterial plasmids replicate by mechanisms involving the generation of theta-shaped, D-looped, or sigma-shaped DNA intermediates. The last mechanism is used by many small multicopy plasmids, and is termed asymmetric rolling-circle (RC) replication because of the uncoupling of leading- and lagging-strand syntheses (reviewed by Novick, 1989; Gruss & Ehrlich, 1989; del Solar et al., 1993a). Initiation of RC-replication is mediated by the plasmid-encoded Rep protein which introduces a strand- and site-specific nick on supercoiled plasmid DNA at the plasmid double-strand origin (dio). Rep activity leaves a 3'-OH end which is extended by host proteins while the parental strand is being displaced, and replication proceeds around the whole plasmid, until the entire dio is synthesized. As a consequence of the termination reactions, the parental (+) strand is released as a single-stranded (ss) DNA intermediate (te Riele et al., 1986), which is the hallmark of RC-replicating plasmids (Gruss & Ehrlich, 1989). The last stage of RC-replication involves the conversion of ss- to ds-plasmid DNA by the synthesis of the lagging strand (Gruss et al., 1987; del Solar et al., 1987). This synthesis initiates in a plasmid region distant from the dio, the single-strand origin, sso (Gruss et al., 1987; del Solar et al., 1987, 1993b). RC-replicating plasmids are constructed like cassettes of genetic information (Projan & Novick, 1988), one of the modules being sso (del Solar et al., 1993a). Based on sequence homologies, various types of sso have been described (Novick, 1989), including ssoA, which is present in plasmids pT181, pLS1 and pC194 (Novick, 1989), and ssoU, which is characteristic of plasmid pUB110 (Boe et al., 1989; Birch & Khan, 1992). A more complex situation is found in the streptococcal plasmid pMV158 (from which plasmid pLS1 is derived; Lacks et al., 1986; Priebe & Lacks, 1989), because it bears both types of sso (van der Lelie et al., 1989). This feature may provide the plasmid with greater flexibility for adapting itself to the different hosts in which it replicates, but poses the question of whether there is preferential lagging-strand origin utilization within a given host.

In general, the sso regions are located within highly structured non-coding regions (Novick, 1989), perhaps with the exception of plasmid pR (Gigliani et al., 1993). The host machinery recognizes the sso in an orientation-dependent manner (Birch & Khan, 1992; del Solar et al., 1993b). These findings suggest that unpaired sequences, within the secondary structures that constitute the sso, are important for its recognition by host proteins involved in
ss → dsDNA conversion. However, it is not clear to what extent these secondary structures contribute to efficient sso function. The mechanisms involved in ss → dsDNA conversion are not well understood, although some features of the process have been defined. First, it seems that only host proteins are involved in the lagging-strand synthesis, most likely RNA polymerase, as shown in vivo for the ssoU of pUB110 (Boe et al., 1989), and in vitro for the ssoA of pT181 (Birch & Khan, 1992). However, in the case of the lactococcal plasmid pWVO1 (an ssoA-containing plasmid) this conversion has been proposed to be mediated by the host DNA primase (Leenhouts et al., 1991). Second, the amount of ssDNA molecules accumulated during RC-replication is plasmid- and host-dependent, indicating that the efficiency of this conversion depends upon the effectiveness with which the host machinery recognizes a given plasmid sso (Gruss et al., 1987; del Solar et al., 1987, 1993b; van der Lelie et al., 1989). However, it is not clear how or where the RNA primer synthesis initiates, and with which specific plasmid sequences it would pair to initiate the lagging-strand synthesis. To answer some of the above questions, we have started a systematic analysis of the regions of the streptococcal plasmid pMV158 involved in ss → dsDNA conversion. We show here that two plasmid elements belonging to ssoA (namely, the recombination site RS_B and a consensus 6 base sequence) contribute to the ss → dsDNA conversion in Streptococcus pneumoniae.

METHODS

Bacterial strains and plasmids. Strains employed for copy number determinations and stability assays were S. pneumoniae 708 (end-1 exo-2 trt-1 boxA malM594) and B. subtilis MB11 (lys-3 metB-10 hsiH-2). Media and growth conditions were as described by Lacks et al. (1986). The plasmids employed, all harbouring the pMV158 replicon and conferring resistance to tetracycline, are listed in Table 1. Selective pressure for the antibiotic was kept at 1 μg ml⁻¹ for S. pneumoniae and at 10 μg ml⁻¹ for B. subtilis. In addition, plasmid pALTER and the Escherichia coli strains from a kit for site-directed mutagenesis (Promega) were used. Copy number determinations were performed as described by del Solar et al. (1987), with the aid of Computer Densitometer-ImageQuant equipment and software (Molecular Dynamics).

DNA preparation and manipulation. Purified plasmid DNAs were prepared from S. pneumoniae by two consecutive CsCl/ethidium bromide gradients as described by del Solar et al. (1987). Total DNA preparations (crude extracts) were made according to published procedures (Lacks et al., 1986). BAL31 deleted derivatives were constructed from plasmid pLS1 linearized at the single Apal site, treated as described by Puyet et al. (1988), and rescued by transformation of the pneumococcal host. The extent of the deletions was analysed by fine restriction mapping and by determination of the nucleotide sequence of the deletion junctions. Enzymes to manipulate the plasmid DNA were purchased from New England Biolabs or from Boehringer Mannheim, and used as specified by the vendors. All constructions were checked by determining the nucleotide sequence of the desired changes using the T7 Sequencing kit (Pharmacia).

Determination of the intracellular amounts of ssDNA. Analyses of ssDNA in cultures harbouring the plasmids of interest were made by electrophoresis on 1% (w/v) agarose gels of total DNA samples, followed by transfer to nitrocellulose filters with or without prior denaturation (et al., 1986). Filters were hybridized using 32P-labelled pLS1 DNA as a probe. When accumulation of ssDNA was measured in cultures treated with rifampicin and/or erythromycin, pneumococcal cultures harbouring the desired plasmids were grown to middle exponential phase (about 2 × 10⁸ c.f.u. per ml culture). Then rifampicin (to 100 μg ml⁻¹), erythromycin (to 100 μg ml⁻¹) or both antibiotics were added. Culture samples were removed at various times and total DNA was analysed as described above. Direct quantification of the amount of ss- and dsDNA transferred to the filters, and calculation of the molecular ratios of ss/dsDNAs, were done with the aid of PhosphorImager-ImageQuant equipment and software (Molecular Dynamics).

Site-directed mutagenesis. The ‘Altered Sites’ kit (Promega), designed for in vitro mutagenesis was used. To perform the mutagenesis, the 1243 bp EcoRI–PstI fragment of pLS1 (coordinates 3170 and 5, respectively; see Fig. 1a) was cloned into pALTER digested with the same enzymes. The consensus sequence 5'-TACCGT-3' of the ssoA was changed into the sequence 5'-TATCGA-3' by the use of the oligonucleotide 5'-CGAGCGGAAAAGGCTTAATGCGACTCCGACGACA- CGGA-3' (underlined letters indicate the mutation introduced within the wild-type sequence). This generates a new ClaI site (5'-ATCGAT-3'), which facilitated the screening of mutated plasmids. The desired mutant plasmid was isolated in E. coli, and the mutation was identified by digestion with ClaI. Then the cloned pLS1 DNA was inserted back into pLS1 by exchanging the EcoRI–PstI fragment. The resulting plasmid (termed pLS1-CM) was rescued by transformation of S. pneumoniae, and the mutation was characterized by determination of its nucleotide sequence.

RESULTS AND DISCUSSION

Deletion mapping of ssoA

The two sso regions of pMV158 are located on the plasmid coding strand. They are separated by the mob gene cassette, involved in plasmid conjugative mobilization (Fig. 1a). The ssoU has been reported to be the preferred lagging-strand origin in B. subtilis (van der Lelie et al., 1989), but its efficiency in the ss → dsDNA conversion had not been analysed in the pneumococcal host. On the other hand, ssoA is efficiently recognized by S. pneumoniae, but not by B. subtilis (del Solar et al., 1987). We had experimentally defined the ssoA region as being between the HindIII and NcoI sites (pMV158 coordinates 4407–5349; Fig. 1a), the fragment removed during construction of plasmid pLS4 (Table 1). Deletion of this 942 bp region in pLS1, which already lacks ssoU, led to plasmids exhibiting an increased accumulation of ssDNA in S. pneumoniae, but not in B. subtilis (del Solar et al., 1987, 1993b). These plasmids, which lack the two sso regions, have a lower copy number, and a high segregational instability in both hosts, with a rate of loss of about 0.1 per cell per generation (del Solar et al., 1993b). We predicted that the ssoA should be included in a region with a high potential to generate
Fig. 1. Relevant features of pMV158. (a) Physical and genetic map of pMV158 and its deleted derivatives. The positions of the dso and the two sso are shadowed. Plasmid-encoded proteins, with direction of synthesis (arrowheads), are indicated. Wavy arrows indicate the position of the two antisense RNAs encoded by pMV158. Only relevant restriction sites are shown. (b) Nucleotide sequence around the ssoA. Coordinates of pMV158 are those by Priebe & Lacks (1989). The recognition sites for AflI and NcoI are underlined. Locations of the recombinase site B (RSb; double underlined), and of the 6 bp consensus (bold) are shown. The borders of the Δ13 and Δ14 deletions and the left border of the Δ24 deletion are indicated. (c) Computer prediction of the secondary structure that could be generated from the sequence shown in (b). Features relevant for this work are indicated.

secondary structures, within which a nuclease-S1-sensitive site has been mapped (del Solar et al., 1987; Fig. 1c). This region is bordered by the restriction sites AflI and NcoI (coordinates 5150 and 5349 of pMV158, respectively; Fig. 1b). Within this putative sso-A-containing region, two conserved sequences have been proposed as being important elements of the lagging-strand origin of replication (Fig. 1c; Gruss et al., 1987; del Solar et al., 1987). The first of these is the recombinase site, termed RSb (Novick et al., 1984), which is involved in recombination and generation of co-integrates among homologous plasmids. This region shares homologies with the pSC101 par locus, which is involved in stable maintenance and which includes a DNA-gyrase-binding site (Wahle & Kornberg, 1988). The second of these, a 6 base consensus sequence (5'-TAGCGT-3'), is located on the terminal loop of the putative hairpin within the ssoA of pLS1, pC221 and pC194 (del Solar et al., 1987; Fig. 1c). This consensus sequence is also present in the ssoA of several other RC-plasmids and coliphages, and has
### Table 1. Features of the plasmids used in this work

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (bp)</th>
<th>Mutation</th>
<th>Relevant features</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMV158</td>
<td>5536</td>
<td>None</td>
<td>Wild-type</td>
<td>Burdett (1980)</td>
</tr>
<tr>
<td>pLS1</td>
<td>4408</td>
<td>ΔEcoRI-B fragment of pMV158</td>
<td>ΔssoU</td>
<td>Lacks et al. (1986)</td>
</tr>
<tr>
<td>pLS1ΔHA</td>
<td>3665</td>
<td>ΔHindIII–AfII of pLS1</td>
<td>ΔssoU</td>
<td>This work</td>
</tr>
<tr>
<td>pLS1ΔNA</td>
<td>4209</td>
<td>ΔAfII–NcoI small fragment of pLS1</td>
<td>ΔssoA, ΔssoU</td>
<td>This work</td>
</tr>
<tr>
<td>pLS1-CM</td>
<td>4408</td>
<td>5 bp change at the ssoA consensus in pLS1</td>
<td>ΔssoU, partially affected in ssoA</td>
<td>This work</td>
</tr>
<tr>
<td>pLS1Δ13</td>
<td>4258</td>
<td>BAL31 deletion of pLS1 linearized at AfII</td>
<td>ΔssoU, partial deletion of ssoA</td>
<td>This work</td>
</tr>
<tr>
<td>pLS1Δ14</td>
<td>4193</td>
<td>BAL31 deletion of pLS1 linearized at AfII</td>
<td>ΔssoU, partial deletion of ssoA</td>
<td>This work</td>
</tr>
<tr>
<td>pLS5</td>
<td>4076</td>
<td>in vivo spontaneous deletion of pLS1</td>
<td>ΔssoU, ΔrnaI (Δ5)</td>
<td>del Solar et al. (1987)</td>
</tr>
<tr>
<td>pLS4</td>
<td>3138</td>
<td>ΔAfII: HIndIII–AfII small fragment of pLS5</td>
<td>ΔssoA, Δ5 (ΔrnaI)</td>
<td>del Solar et al. (1987)</td>
</tr>
<tr>
<td>pLS4-EC</td>
<td>4266</td>
<td>ΔAfII, EcoRI-B fragment of pMV158 cloned in pLS4 (correct orientation)</td>
<td>ΔssoA, Δ5 (ΔrnaI)</td>
<td>This work</td>
</tr>
<tr>
<td>pLS4-EI</td>
<td>4266</td>
<td>ΔAfII, EcoRI-B fragment of pMV158 cloned in pLS4 (incorrect orientation)</td>
<td>ΔssoA, Δ5 (ΔrnaI)</td>
<td>This work</td>
</tr>
<tr>
<td>pLS1Δ24</td>
<td>3838</td>
<td>BAL31 deletion of pLS1 linearized at BanI</td>
<td>ΔssoU, ΔrnaI</td>
<td>A gift from P. Acebo</td>
</tr>
</tbody>
</table>

**Fig. 2.** Location of regions important for a functional ssoA. In addition to pMV158, plasmids derived from pLS1 (ΔssoU) were employed. The intracellular ssDNA accumulated by the indicated plasmid derivatives was detected by Southern blot hybridization of total DNA isolated from plasmid-containing cultures and electrophoresed in 1% agarose. DNA was transferred after denaturation, so that ds- and ssDNA are visible. The positions of ssDNA in *S. pneumoniae* (a) and in *B. subtilis* (b) are bracketed. The leftmost lanes in part (a) are overexposed to visualize the ssDNA.

been used as a criterion for the location of lagging-strand origins (Zaman et al., 1993). Part of the RSB as well as the consensus sequence are located within unpaired regions within the *ssoA* (Novick, 1989).

To define more precisely the boundaries of the *ssoA*, AfII-linearized pLS1 DNA was treated with BAL31 nuclease. Deleted pLS1-derivatives were rescued by transformation of *S. pneumoniae*, and several of them were
characterized. Two plasmids were selected, both of them lacking the RS_B but preserving the consensus sequence (pLS1A13 and pLS1A14; Fig. 1). Plasmids lacking the entire AflII-NeoI fragment (pLS1ANA), or the HindIII-AflII region (pLS1AHA) were also constructed (Table 1). As controls, pMV158 (wild-type), pLS1 (AssoU), pLS1A24 (AssoU, AssoA) and pLS4 (AssoU, AssoA) were used (Fig. 1a). All these plasmids were transferred to S. pneumoniae and to B. subtilis, and the ssDNA accumulated was detected (Fig. 2), and quantified (Table 2). In the pneumococcal host (Fig. 2a), no influence of the HindIII-AflII region in accumulation of ssDNA was observed, since the plasmid pLS1AHA accumulated as little ssDNA as pLS1 or pMV158. In addition, sequences located at 3' of the NcoI site (on the coding strand, Fig. 1b) do not play any role in the ss → dsDNA conversion, since the amount of ssDNA detected for pLS1A24 was similar to that of pLS1 (Fig. 2a). However, a 15-fold increase in the amount of ssDNA was detected in plasmid pLS1A13, with respect to pLS1, and a further twofold increase was found in pLS1A14. Deletion of the whole AflII-NeoI region (pLS1ANA) further increased the amount of ssDNA accumulated. These results demonstrate that the sso_A of plasmid pMV158 is located within the coordinates 5150 and 5349, which experimentally narrows down this lagging-strand origin from 942 bp (del Solar et al., 1993b) to a 199 bp region (Fig. 1b). Regions similar in size have been defined for the sso_A region of pLS1A24 was similar to that of pLS1 (Fig. 2a). However, a 15-fold increase in the amount of ssDNA was detected in plasmid pLS1A13, with respect to pLS1, and a further twofold increase was found in pLS1A14. Deletion of the whole AflII-NeoI region (pLS1ANA) further increased the amount of ssDNA accumulated. These results demonstrate that the sso_A of plasmid pMV158 is located within the coordinates 5150 and 5349, which experimentally narrows down this lagging-strand origin from 942 bp (del Solar et al., 1993b) to a 199 bp region (Fig. 1b). Regions similar in size have been defined for the sso_A region of pLS1, could play a role as part of this conversion signal (del Solar et al., 1987). To determine whether the consensus is involved in the ss → dsDNA conversion, we changed five bases of this sequence without significantly altering intrastand pairing within the secondary structure (Fig. 3a). Due to the presence of a T just before the consensus, the mutation generated a weak consensus-like sequence 5'-TATCGA-3' (Fig. 3a). Curiously, this sequence has only one mismatch (the central T) as compared with the consensus found at the sso_A of plasmid pE194 (del Solar et al., 1987). Some of the features of the mutant plasmid, termed pLS1-CM, were analysed only in S. pneumoniae because of the lack of functionality of sso_A in B. subtilis. The phenotype of pLS1-CM was indistinguishable from its parental pLS1 with respect to copy number (Table 2). To evaluate statistically accumulation of ssDNA in cells harbouring pLS1-CM, eight independent DNA preparations were analysed. The results (Table 2, Fig. 3b) showed that a significant 26-fold increase in the amount of ssDNA was observed within cells harbouring pLS1-
M. G. KRAMER, G. DEL SOLAR and M. ESPINOSA

CM, as compared with pLS1 or pMV158. These results allowed us to conclude that the consensus sequence by itself may play a role within the conversion signal. However, we cannot rule out that the putative configuration of the loop of the ssoA, which would be larger in the mutant than in the wild-type (Fig. 3a), could influence the ss → dsDNA conversion.

**ssoU is efficiently recognized by S. pneumoniae**

Pneumococcal cells harbouring pMV158 accumulate as small an amount of ssDNA as pLS1 (Fig. 2a), which could be due solely to the presence of the functional ssoA. To test whether ssoA could be replaced by the ssoU in S. pneumoniae, the 1128 bp EcoRI fragment B of pMV158, which is removed in pLS1 and its derivatives (Fig. 1a), was cloned into the single EcoRI site of plasmid pLS4 in the two possible orientations (Table 1). If the ssoU acts as an efficient lagging-strand origin in pneumococci, low amounts of ssDNA should be detected only when the EcoRI-B fragment is cloned in the correct (pLS4-EC), but not in the opposite (pLS4-EI), orientation. This was indeed the case, and pLS4-EC accumulated little ssDNA, whereas pLS4-EI behaved similarly to its parental pLS4 (Fig. 4a). In addition, the decrease in copy number (measured as dsDNA) observed for pLS4 was restored to the levels of pLS1 only in the case of pLS4-EC (Table 2). Similar results on ssDNA accumulation were found for B. subtilis (Fig. 4b), although the number of copies of the plasmids did not vary (Table 2). This finding supports previous observations indicating that the ssoU is the functional lagging-strand origin in B. subtilis (van der Lelie et al., 1989). We can thus conclude that the pMV158-ssoU acts as an efficient conversion signal in both hosts. It remains to be shown whether the two sso present in pMV158 play different roles in S. pneumoniae. Due to its location within the plasmid (Fig. 1a), we can speculate that the ssoU would be the lagging-strand origin preferentially used after conjugative plasmid transfer, since deletion of a region which encompasses the ssoU resulted in loss of the mobilization function (Priebe & Lacks, 1989). In this sense, it is curious that the two sso regions are placed just before and just after the mob gene. Alternatively, RC-replication from the dre would lead to the exposure of ssoU as an ssDNA region before the replisome reaches the ssoA. If the host machinery follows a 'first-exposed, first-used' pattern for ss → dsDNA conversion, then ssoU should be the preferential lagging-strand origin employed in pMV158.

**Pneumococcal RNA polymerase is involved in the ss → dsDNA conversion**

RC-plasmids pUB110 and pT181 require the host RNA polymerase for ss → dsDNA conversion (Boe et al., 1989; Birch et al., 1992). To determine whether pMV158 uses the S. pneumoniae RNA polymerase for lagging-strand synthesis, exponentially growing cells harbouring pLS1 (AssoU), pLS4-EC (AssoA) or pLS4 (AssoU AssoA) were subjected to inhibition of RNA and/or protein synthesis, by treatment with rifampicin, erythromycin or both antibiotics simultaneously. Rifampicin treatment would prevent RNA polymerase activity, whereas inhibition of protein synthesis would hinder synthesis of the initiation of replication RepB protein, thus preventing (at least partially) further rounds of replication from the plasmid dre. The amount of plasmid DNA was quantified and the molecular ratio of ss/dsDNA was plotted as a function of the time of treatment (Fig. 5). A 10-fold increase in this ratio was observed in the cultures in which RNA or RNA and protein synthesis were inhibited, but only if they harboured plasmids having one or another sso (Fig. 5a, b). A net increase in the amount of ssDNA accumulated upon inhibition of RNA polymerase could be observed in either pLS1 and pLS4-EC, but not in pLS4 (Fig. 6). No such accumulation was found for the sso-containing plasmids in the absence of antibiotics (Fig. 6). Consequently, the accumulation of ssDNA after inhibition of RNA synthesis (but not of protein synthesis) suggests that an active RNA polymerase is needed to avoid accumulation of ssDNA within the cells. This in turn indicates that the pneumococcal RNA polymerase is the enzyme involved in priming DNA synthesis from both lagging-strand origins. These results are supported by the observation that no significant changes were observed for the sso-deleted plasmid pLS4 (Fig. 5c). We conclude that the same host enzyme is involved in synthesis of a primer in two plasmid regions located far apart, and apparently performing the same function. The ssoA and the ssoU regions exhibit high potential to generate secondary structures (data not shown) and, since both sso regions are recognized by the host RNA polymerase, we expected to find some sequence conservation among them. However, computer searches
Replication from lagging-strand origins

Fig. 5. Influence of rifampicin on ss → dsDNA conversion. Pneumococcal cultures harbouring pLS1 (ΔssoU, panel (a)), pLS4-EC (ΔssoA, panel (b)) or pLS4 (ΔssoA ΔssoU, panel (c)) were treated with rifampicin (△), erythromycin (○) or both antibiotics (●). At the indicated times, samples were removed, and the molecular ratios of ss/dsDNA accumulated were quantified.

Fig. 6. Intracellular accumulation of ssDNA. Pneumococcal cultures harbouring the indicated plasmids were treated (+) or not treated (−) with rifampicin or erythromycin as in Fig. 5. Samples were removed at various times, and total DNA was analysed as in Fig. 2. The position of ssDNA is indicated.

showed neither significant homologies between the ssoU and the ssoA, nor any putative promoter-like sequence (data not shown). Plasmids having a low G+C content, like pMV158, do have several sequences identical to the promoter −10 consensus region, but their significance is questionable. Consequently, which DNA signals are recognized by the RNA polymerase is unknown.

An interesting finding is that, in spite of the high amount of ssDNA accumulated in cells harbouring plasmids devoid of sso, those plasmids could be maintained under selective pressure. This indicates that at least an alternative pathway for the conversion of ss → dsDNA should exist. How then could plasmids with defective lagging-strand origins replicate? Based upon indirect evidence, we
proposed that an alternative sso could exist within the tet gene of pMV158 (del Solar et al., 1987). If this alternative sso is functional, a host function, other than RNA polymerase, and insensitive to inhibition of protein synthesis, should be involved in this weak mechanism of ss → dsDNA conversion. Based on our results, we believe that both sequence-specific elements (RS$_R$ and 6-mer consensus) and secondary structures could be the best combination for a region to function as an sso. The results obtained so far with pMV158 allow us to draw the following sequence of events in the ss → dsDNA conversion process. Lagging-strand synthesis would initiate from an ssDNA plasmid template which should be covered by the host single-stranded DNA binding protein, except in those regions having intrastrand pairing. Secondary structures, in conjunction with paired and unpaired sequence-specific sites, could act as signals which would be recognized by the host RNA polymerase. Upon synthesis of an RNA primer, DNA polymerase III would proceed to synthesize DNA until reaching an sso which would now be a DNA–RNA hybrid. The last stage would be the removal of the RNA and termination of DNA synthesis, which is performed by the host DNA polymerase I (Diaz et al., 1994).

ACKNOWLEDGEMENTS

Thanks are due to M. T. Alda, P. Valiente and R. Galán for technical assistance. Corrections to the English by D. M. Main are very much appreciated. Research was financed by CICYT (Grant BIO94–1029), and Comunidad Autónoma de Madrid (Grant 190/92). M.G.K. is the recipient of a Celestino Mutis fellowship from the Agencia Española de Cooperación Internacional.

REFERENCES


Received 16 September 1994; revised 25 October 1994; accepted 28 October 1994.