Sequence Requirements for Protein-primed Initiation and Elongation of Phage Ø29 DNA Replication*

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The double-stranded linear DNA of Bacillus subtilis phage Ø29 is replicated by a mechanism in which a terminal protein (TP) acts as a primer. The second 3'-terminal nucleotide of the template directs the incorporation of the 5'-terminal nucleotide into the TP, giving rise to the initiation complex TP-dAMP. Elongation then proceeds by a sliding-back mechanism in which the dAMP covalently linked to the TP pairs to the 3'-terminal nucleotide of the template strand to recover full-length DNA. We have studied the sequence requirements for efficient initiation of replication using mutated TP-free double-stranded DNA fragments. Efficient initiation only requires the terminal repetition 5'-AA. The 3'-terminal T, although not used as template, increases the affinity of DNA polymerase for the initiator nucleotide; in addition, although to a minor extent, the third 3'-terminal position also directs the formation of the initiation complex and modulates the initiation rate at the second position. Efficient elongation requires a previous sliding-back, demanding again a repetition of two nucleotides at the 3' end; if the sliding-back is prevented, a residual elongation can proceed directly from the second position or after jumping back from the third to the first position.

DNA replication requires a primer to provide the hydroxyl group that all DNA polymerases need to initiate DNA synthesis (reviewed in Ref. 1). Many linear genomes that replicate from their ends use a protein, the so-called terminal protein (TP), as a primer by forming a phosphodiester linkage between the OH group of an amino acid residue and the 5'-terminal nucleotide. Such TP-containing genomes have been found in bacteriophages (e.g. Ø29, PRD-1, Cp-1), eukaryotic viruses (e.g. adenoviruses), linear plasmids (e.g. S1, pGKL1), and bacteria (e.g. Streptomyces), and most of them have inverted terminal repeats (ITR) with sizes ranging from 6 bp, like adenoviruses, that is specifically recognized by the DNA polymerase I of initial DNA synthesis (6). In the case of the extensively studied protein-primed replicon of bacteriophage Ø29, the parental TP is an active component of the replication origins located at both genome ends that contain the ITR 5'-AAAGTA, specifically recognized by the replication machinery formed by the primer TP/DNA polymerase heterodimer (7, 8). However, TP-free Ø29 DNA terminal fragments retain about 10–15% of the template activity of TP-containing fragments (8–10). By deletion analysis of TP-free DNA fragments containing Ø29 terminal sequences, it was proposed that the minimal origins of replication are comprised within the terminal 12 bp, either from the right or left DNA ends (11).

Escherichia coli phage PRD1 also has a linear genome that replicates by a protein-priming mechanism. It has been reported that, using TP-free fragments as templates, the terminal 20 bp of the 110-bp-long ITR are required for efficient in vitro DNA replication (12). The existence of a precise DNA sequence, required for efficient template recognition, has been also proposed for bacteriophage Cp-1 from Streptococcus pneumoniae (13). Similarly, the terminal 18 bp of adenovirus genome represents the minimal replication origin and contains a 10-bp region (positions 9–18), conserved in all human adenoviruses, that is specifically recognized by the DNA polymerase complexed with the preterminal protein (Ø29 TP counterpart) (14).

Using single-stranded oligonucleotides as templates, it was demonstrated that the 3’ second nucleotide, with no significant sequence specificity, directs the incorporation of the complementary dNTP into the primer TP to form the initiation complex TP-dNMP (15). Full-length Ø29 DNA replication requires the pairing of the initiator nucleotide to the 3'-terminal nucleotide of the template. This mechanism, named sliding-back, needs a terminal repetition of 2 bp and provides a mechanism to prevent mutations at the Ø29 DNA ends (15), since the 3’ exonuclease activity of Ø29 DNA polymerase cannot proofread the TP-linked nucleotide (16).

In other protein-primed linear genomes, similar situations have been described; thus, in the case of Ø29-related bacteriophage GA-1 (5'-AAAA), initiation also occurs at the 3’ second nucleotide of the template and, to a lesser extent, at the third nucleotide (17). S. pneumoniae phage Cp-1 initiates at the 3’ third nucleotide of its terminal repetition (5’-AAAA) (13), and E. coli phage PRD1 initiates at the fourth nucleotide (5’-GGGG) (18) as well as adenoviruses, with a 5’-CATCAT reiteration (19). The sliding-back mechanism of elongation seems to be a common feature of protein-priming systems to restore full-length DNA. In the case of phage GA-1, the residual initiation directed by the third nucleotide is proposed to jump-back pairing to the first nucleotide (17). However, for phages Cp-1 and

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PRD1, initiating, respectively, at the third and fourth nucleotide, a stepwise sliding-back mechanism that recovers the preceding nucleotides sequentially, is favored (13, 18). Adenovirus initiation complex elongates from the fourth to the sixth position, and then the TP-CAT is aligned with the three terminal bases of the template by a jumping-back mechanism (19).

In this work we have further characterized the minimal sequence at the replication origin of Ø29 DNA required for an efficient initiation and elongation. We have found that conservation of the terminal repetition 5′-AA is necessary for the optimal formation of the initiation complex TP-dNMP. In particular, the 3′-terminal nucleotide, which is not used as template, modulates the initiation efficiency, impairing that of mutated templates. This checkpoint would provide a mechanism to preserve the nucleotide sequence of the genome ends.

MATERIALS AND METHODS

Nucleotides, Oligonucleotides, and Proteins—Unlabelled and α-32P-labeled (3000 Ci/mmol) dNTPs as well as micrococcal nuclease were purchased from Amersham Pharmacia Biotech. Single-stranded oligonucleotides were from Isogen. Proteinase K was from Roche Molecular Biochemicals. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and Vent DNA polymerase were from New England Biolabs. Calf intestinal alkaline phosphatase was purchased from Promega. Ø29 DNA polymerase was obtained from E. coli NF2690 cells harboring plasmid pJLw2 and purified as described (20); mutant T15I of Ø29 DNA polymerase was obtained as described (21). Ø29 TP was purified from E. subtilis harboring a TP-expressing plasmid (22).

TP-free DNA Fragments—Proteinase K-digested Ø29 DNA was purified as described (23). Wild-type or mutated Ø29 DNA left terminal fragments (259 bp) were obtained by polymerase chain reaction amplification from proteinase K-digested Ø29 genome by using the appropriate oligonucleotides and directly purifying the polymerase chain reaction products. The name of mutated ori L9 indicates the 5′-terminal sequences of template DNAs. Fig. 1 shows the 5′-terminal sequences of template DNAs.

RESULTS

Initiation of Replication Activity of TP-free Templates Requires a Terminal Repetition of Two Nucleotides—TP-free Ø29 DNA terminal fragments can be used as templates for initiation of DNA replication, although their activity is 10–15% that of TP-DNA (8–10). The improvement of the Ø29 DNA replication in vitro system together with the use of Mn2+, a 100-fold better activator of protein-primed initiation than Mg2+ (24), allowed us to carry out a systematic study on the ability of TP-free double-stranded DNA fragments to act as templates for initiation of DNA replication. Plasmid pL259, containing the left terminal 259 bp of Ø29 DNA (ori L), was digested with different restriction enzymes, giving rise to a collection of blunt-ended fragments with different numbers of Ø29-conserved terminal nucleotides. Fig. 1 shows the 5′-terminal sequences of the template complementary strands and the conserved nucleotides with respect to ori L. Fragments are named after the number of conserved terminal nucleotides. Fragment L1* conserves the second nucleotide instead of the terminal nucleotide. The fragments were very similar in size, in order to minimize the described effect of DNA length in the in vitro activity (10). The sequences at the other end of the fragments do not allow the formation of the TP-dAMP initiation complex. Therefore, the initiation activity, shown in Fig. 2, can only correspond to the ends shown in Fig. 1.

As shown in Fig. 2, fragments L5, L3, and L2 showed an activity comparable with that of the ori L control, specially at early reaction times. However, fragments L1 and L0 behaved as the background, with no template. The latter result was expected, since it was shown that the second 3′-terminal nucleotide directs the incorporation of the initiator nucleotide to primer TP in single-stranded oligonucleotides (15), and in fragments L1 and L0, they are not complementary. However, fragment L1* contains the template nucleotide complementary to the initiator nucleotide, yet it was equally inactive. All these results were qualitatively the same when Mg2+ was used instead of Mn2+, so the nature of the metal seems to have no effect in sequence requirements (not shown). These results suggest that the template nucleotide by itself is not enough to direct the formation of the initiation complex and that the first
and second 3'-terminal nucleotides of the template are critical for efficient initiation of replication, whereas the rest of the terminal sequence would have, if any, a minor role.

**The 3'-Terminal Nucleotide Is Not Used as Template but Modulates Initiation**—The lack of activity observed with fragment L1* could be explained if initiation were directed by the 3'-terminal nucleotide. However, this is not consistent with the result obtained with fragment L1 (see Fig. 2) and with those with single-stranded oligonucleotides (15). Alternatively, the initiation directed by the second 3'-terminal nucleotide could be modulated by the first nucleotide. To address directly these questions and to avoid the effect of more internal sequences, we used as templates ori L fragments containing single-point mutations obtained by polymerase chain reaction. The possibility of initiation directed by the 3'-terminal nucleotide was tested using the mutants ori L CAA, ori L GAA, and ori L TAA, in which the 5'-terminal nucleotide, A, complementary to the ori L template was changed to C, G, and T, respectively. The initiation assays were performed with the dNTP complementary to the 3'-terminal nucleotide (Fig. 3A). These results were compared with the misincorporation background, in which ori L was assayed with each of the three dNTPs (Fig. 3B); the values for ori L CAA, ori L GAA, and ori L TAA were 40, 75, and 60%, respectively, that of the corresponding misincorporation background (Fig. 3, A and B). Since the activity of the three mutated origins with the dNTP complementary to the 3'-terminal nucleotide was lower than the misincorporation obtained with ori L, we definitively ruled out the possibility of initiation directed by the terminal nucleotide of the template.

The influence of the terminal nucleotide on the formation of the initiation complex was tested with the same templates but with dATP as initiator nucleotide. Fig. 4 shows that the highest activity was obtained by the wild-type ori L, with ori L TAA, ori L GAA, and ori L CAA at 41, 23, and 18%, respectively, after 20 min of reaction. Therefore, mutations at the first position decrease the rate of initiation. However, this observation could be explained either by a preferential incorporation when T is at the 3' end or when the two terminal nucleotides are the same, which is the requirement for the sliding-back mechanism for elongation. Fig. 5 shows that ori L still is the most active template when compared with double mutants ori L CCA, ori L...
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GGA, and \( \text{ori L} \) TTA, providing in each case the nucleotide complementary to the second 3'-terminal nucleotide. Moreover, with dCTP as initiator nucleotide, the mutated template \( \text{ori L} \) ACA is about 2.5-fold more active (not shown) than \( \text{ori L} \) CCA, supporting the notion that a 3'-terminal T, although not used as template, favors the initiation reaction, regardless the nature of the initiator nucleotide. This could be achieved by increasing the affinity of DNA polymerase for the incoming dNTP. In fact, the \( K_m \) for dATP with wild-type \( \text{ori L} \) ACA is about 10 times for \( \text{ori L} \) CCA, whereas for \( \text{ori L} \) TAA, \( \text{ori L} \) CAA, and \( \text{ori L} \) GAA it is 10, 17, and 28 \( \mu \text{M} \), respectively (not shown).

The Third 3'-Terminal Nucleotide Is Also Used as an Initiation Site—By using single-stranded oligonucleotides, it has been suggested that the third 3'-terminal nucleotide could direct, although at a smaller extent than the second nucleotide, the incorporation of the initiator nucleotide into the TP (15). To evaluate the contribution of the third 3'-terminal nucleotide in the initiation activity of \( \Phi 29 \) double-stranded DNA templates, we used \( \text{ori L} \) single mutants at the third 3'-terminal position to assay their initiation activity with the corresponding complementary dNTPs. Fig. 6 shows the initiation activity of \( \text{ori L} \) AAG, \( \text{ori L} \) AAT, and \( \text{ori L} \) AAC. In all cases, the activity is higher than the misincorporation background (see Fig. 3B): 4.7 times for \( \text{ori L} \) AAG, 6.2 times for \( \text{ori L} \) AAT, and 6.4 times for \( \text{ori L} \) AAC, indicating that the third 3'-terminal nucleotide specifically directs TP-dNMP formation. Therefore, both the second and third 3'-terminal nucleotides could be used as template initiation sites for the formation of the TP-dNMP, and the activity observed in \( \text{ori L} \) wild type would include initiation from both positions. We can measure the initiation only from the second position using the same templates with dATP as initiator nucleotide. As Fig. 7 shows, the activity varies with the third 3'-terminal nucleotide; thus, the activity of \( \text{ori L} \) AAG, \( \text{ori L} \) AAT, and \( \text{ori L} \) AAC was, after 20 min of reaction, 66, 55, and 27%, respectively, that of the wild type. Since we cannot measure independently initiations directed from the second or the third positions of the wild-type template, we cannot distinguish to what extent the observed drop in TP-dAMP formation is due to the lack of initiation at the third template position or to a decrease in the initiation at the second template position modulated by the third 3'-terminal nucleotide. In general, mutations at the 3'-terminal position have a greater effect than those at the third position, despite the fact that the first position only modulates the initiation reaction, whereas the third position is used also as initiation site (see Figs. 4 and 7). When both positions are mutated there is a cumulative effect, and the activity drops, as seen by \( \text{ori L} \) TAT that has 30% wild-type activity (not shown). A more drastic effect is shown by \( \text{ori L} \) GAC, with only 6% activity (not shown) as compared with \( \text{ori L} \) GAA (23%, see Fig. 4) and \( \text{ori L} \) AAC (27%, see Fig. 7). Interestingly, the activity retained by \( \text{ori L} \) AAT (55%, see Fig. 7) and \( \text{ori L} \) GAC (6%, not shown) is similar to that of fragments L2 and L1* (49 and 1%, respectively, see Fig. 2), which also have AAT and GAC at their 5' termini but followed by a different sequence (see Fig. 1). These results further suggest that the nucleotide sequence beyond the third 3'-terminal position has a negligible influence in the initiation activity.

A Terminal Repetition of Two Nucleotides Is Required for
Efficient Elongation of the Initiation Complex—The initiation at the second (or third) position of the template in Φ29 DNA replication requires a sliding-back mechanism for elongation to preserve the size of the DNA (15). To study the nucleotide sequence requirements for elongation, we have used the ori L mutants as templates in a truncated elongation assay, where all the dNTPs, except dCTP, are supplied, forcing DNA polymerase to stop replication after the incorporation of the eighth nucleotide to the nascent chain (see Fig. 8). The exonuclease-free T15I mutant Φ29 DNA polymerase (21) was used to prevent degradation of elongation products. We found that a terminal repetition of only 2 bp is enough for an efficient truncated elongation, in agreement with the results obtained for single-stranded oligonucleotides (15). As shown in Fig. 8A, elongation products of ori L AAG and ori L AAT have the same length as that of the wild type (the sliding-back elongation mechanism of ori L AAT is illustrated in Fig. 9). Furthermore, sliding back takes place regardless the nature of the nucleotides, as shown by the ori L GGA template. The higher mobility bands correspond to the initiation complex TP-dAMP and TP-(dAMP) 2, as indicated. They are not seen with ori L GGA, since the only labeled nucleotide is dATP, and the initiation at the third 3′-terminal position in this template is particularly low (7%, not shown).

A mutation at the 3′ end of the template would prevent the sliding back of complexes initiated at the second position. Fig. 8B shows that elongation is highly impaired in 3′-terminal mutants; however, the residual elongation products obtained with ori L CAA, ori L GAA, and ori L TAA are one nucleotide shorter than those obtained with the wild-type sequence. These results could be due to initiation at the second position followed by elongation with no sliding back or elongation of complexes initiated at the third position that slide back to the second position. To test the possibility of elongation without sliding back, we assayed for truncated elongation the mutated fragments ori L TAT and ori L AGA, in which sliding back from the third to second positions is not allowed (Fig. 8C). Again, elongation was strongly diminished, and the products observed corresponded to TP-(dNMP) 2, suggesting that initiation at the second position may be followed by elongation without previous sliding back (the mechanism of elongation for ori L TAT is illustrated in Fig. 9). Interestingly, when ori L ATA was used as template, we detected a faint band corresponding to the full-length truncated elongation product. Here, full-length recovery is only possible if an initiation at the third position is followed by a jumping back to the first position (see Fig. 9). This mechanism is consistent with the previous result that the third 3′-terminal position of the template can be used as initiation site (see Fig. 6). The lack of a full-length elongation product in ori L AGA and ori L TAT could be explained by their low initiation rate at the third 3′-terminal position, 3–5 times lower than in ori L ATA (not shown).

In summary, efficient elongation of Φ29 protein-primed initiation complexes requires at least a 3′-terminal repetition of two nucleotides to slide back. At a much lower extent, elongation can proceed with no sliding back or, in some cases, by jumping back from the third to the first 3′-terminal template positions. We cannot rule out elongation of complexes initiated at the third 3′ position that slide back to the second position.

**DISCUSSION**

Phage Φ29 DNA replication origins are located at both ends of its linear double-stranded genome and are characterized by the presence of a TP covalently linked to each 5′ end (1). Although TP plays a crucial role in the origin recognition by the replication machinery, TP-free Φ29 DNA terminal fragments retain about 10–15% of their template activity in protein-primed initiation of replication (8–10). Deletion analysis suggested a minimal origin of replication within the terminal 12 bp at each end; however, point mutations at the conserved positions did not affect their template activity except those at the second and third terminal positions, in which they were completely abolished when dATP was used as initiator nucleotide (11). These results became clear when, by using single-stranded oligonucleotides as templates, it was found that ini-
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Fig. 9. Mechanisms of elongation in ori L mutants. Three elongation mechanisms, corresponding to three ori L templates, are illustrated. The ori L AAT mutant elongates by the same sliding-back mechanism as the wild-type ori L. The ori L TAT mutant elongates with no sliding back. The ori L ATA mutant elongates by a jumping-back mechanism.

| Template | ori L AAT | ori L TAT | ori L ATA |
|----------|-----------|-----------|-----------|
| Product  | TP-(dNMP)_8 | TP-(dNMP)_7 | TP-(dNMP)_8 |

The initiation of replication is directed by the second instead of the 3'-terminal nucleotide of the template (15). The selection of the template initiation site is determined by the DNA polymerase-TP complex rather than the nucleotide sequence. Thus, the same Ø29 template is initiated at the third 3'-terminal position by the phage Cp-1 complex (13) and at the second position by the Ø29 complex (15).

We have further studied the role of the terminal nucleotide sequence in the efficiency of the initiation of replication by using TP-free double-stranded DNA fragments and a more active in vitro system in which Mn²⁺ is used as metal activator. The results obtained with fragments sharing a variable number of terminal bp with Ø29 ori L showed that the terminal repetition 5'-AA is the only requirement for an efficient initiation complex formation, ruling out a specific sequence recognition by the replication machinery. Our finding that a fragment that had the second, but not the first, terminal nucleotide conserved (fragment L1*, see Fig. 2) did not initiate replication, suggested a role for the 3'-terminal nucleotide. Furthermore, ori L CAA, ori L GAA, and ori L TAA showed reduced initiation activities (Fig. 4). We have ruled out the possibility that the first position is used as template for the formation of the initiation complex, since there is no specific incorporation of the dNMP complementary to the first position of mutated ori Ls (Fig. 3). There is no requirement for a mere terminal repetition of two nucleotides, as indicated by the activity of ori L CCA (7%), ori L GGA (22%), and ori L TTA (47%); these results cannot be explained on the different intrinsic affinity of Ø29 DNA polymerase for each dNTP in the initiation reaction, measured in the absence of template: 170% for dTTP, 63% for dGTP, and 22% for dCTP (100% for dATP) (25). Altogether our results indicate that the 3'-terminal nucleotide, although not used as template, strongly influences the initiation complex formation directed by the second terminal nucleotide. The highest activity is obtained with a 3'-terminal T, regardless the nature of the initiator nucleotide; thus, ori L ACA was 2.4-fold more active than ori L CCA (not shown). Single mutations at the 3' end increase the K_m value for dATP-DNA (16). Thus, the 3'-terminal T of the template together with the 5'-linked parental TP increase the affinity of DNA polymerase for the initiator nucleotide, which is selected by the second position of template.

A similar result was observed with single-stranded oligonucleotides as templates, in which 3'-CTT showed a 30% initiation efficiency of the wild-type 3'-TTT (26). In other protein-primed replication systems such as S. pneumoniae phage Cp-1, in which the TP-linked nucleotide is selected by the third template nucleotide, a T to C mutation at the 3'-terminal position drops the initiation efficiency to 34% and below 1%, when the mutation is at the second position (13). In E. coli phage PRD1, the initiation site is at the fourth template nucleotide; single-stranded oligonucleotides 3'-CTCC and 3'-CCTC show 11 and 52% wild-type 3'-CCCCC initiation activity (18).

The fact that mutations of the nucleotides preceding the initiation site result in decreased initiation of replication is probably related to a structural requirement of the DNA polymerase active site, either to stabilize the initiator nucleotide and/or to form the covalent bond with TP. The conformation of the active site determines the efficiency of DNA polymerase to incorporate the correct nucleotide on the basis of steric (geometrical and electrostatic) constricctions rather than on the mere facility to form hydrogen bonds (27, 28). The biological significance of the initiation impairment of templates mutated at the 3'-terminal position could be to preserve the Ø29 genome ends, as Ø29 DNA polymerase cannot proofread the 5'-terminal nucleotide (16). Thus, this would be an additional mechanism to that described for the sliding back, required for elongation of the initiation complex (15).

In addition, there is a significant initiation directed by the 3rd 3'-terminal nucleotide of the template, as shown by the experiments with the single mutants ori L AAC, ori L AAG, and ori L AAT (Fig. 6). A residual initiation at the third position had been detected in some cases in single-stranded oligonucleotides with Ø29 (15) and at a higher level with the related phage GA-1 (17). We cannot measure independently the initiation directed by the second and third positions in wild-type ori L. However, the initiation activity with dATP of ori L AAC, ori L AAG, and ori L AAT is lower than that of wild-type ori L and depends on the nature of the third 3' nucleotide. Therefore, the initiation directed by the second 3'-terminal nucleotide is not only influenced by the first but also, to a lesser extent, by the third nucleotide, as the activity of the mutants at the third position is higher than that of the mutants at the first position (see Figs. 4 and 7), even though the latter can use the second and third positions as template.

It has been described that full-length elongation is achieved by a sliding-back mechanism from the second to the first template positions (15). We have also used point mutations of ori L fragments to study the template requirements for truncated elongation. As it was described previously for single-stranded templates (15), full-length elongation takes place with tem-
plates with a terminal repetition of two nucleotides (Fig. 8A) to an extent similar to that of the wild-type ori L. This is also true when the terminal repetition is not 5'-AA. However, elongation is strongly impaired when the sliding back is prevented, as is the case of first position-mutated ori Ls (Fig. 8B). The low initiation rate of these mutants does not fully account for their poor elongation. In these cases the elongation products are one nucleotide shorter, ruling out a mismatched sliding back. The latter possibility was demonstrated for initiation at the second position followed by a sliding back to the second position and/or to an initiation at the second position followed by elongation with no sliding back. The latter possibility was demonstrated with mutants in which any sliding back is prevented, such as ori L TAT and ori L AGA, which again give rise to elongation products one nucleotide shorter than full-length. In contrast, ori L ATA produced a full-length elongate, suggesting that initiation at the third position (3–5-fold higher than that of ori L TAT and ori L AGA) is followed by a jump back to the first position. A similar jumping-back mechanism has been suggested for Ø29-related phage GA-1 (17). In adenovirus, initiation occurs at the third position, and elongation proceeds to the sixth, then by jumping back, aligns the trinucleotide to the three 3'-terminal positions (19).

In conclusion, we have determined the sequence requirements for efficient replication of Ø29 DNA at the stages of initiation and elongation. In addition to the 3' second T at the 3' end, the third T is also used as template for TP-dAMP synthesis. More interestingly, the 3'-terminal T, although not used as template, is required for efficient initiation, providing a mechanism to prevent mutations at the Ø29 genome ends.

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