Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 9579–9583, October 1992
Biochemistry

Initiation of ϕ29 DNA replication occurs at the second 3′ nucleotide of the linear template: A sliding-back mechanism for protein-primed DNA replication

(bacteriophage ϕ29/initiation site/linear DNA replication/mutated origins)

JUAN MÉNDEZ, LUIS BLANCO, JOSÉ A. ESTEBAN, ANTONIO BERNAD, AND MARGARITA SALAS†

Centro de Biología Molecular (Consejo Superior de Investigaciones Cientificas-Universidad Autónoma de Madrid), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

Communicated by Severo Ochoa, June 5, 1992

ABSTRACT **Bacteriophage ϕ29 DNA replication is initiated when a molecule of dAMP is covalently linked to a free molecule of the terminal protein, in a reaction catalyzed by the viral DNA polymerase. We demonstrate that single-stranded DNA molecules are active templates for the protein-primed initiation reaction and can be replicated by ϕ29 DNA polymerase. Using synthetic oligonucleotides, we carried out a mutational analysis of the ϕ29 DNA right end to evaluate the effect of nucleotide changes at the replication origin and to determine the precise initiation site. The results indicate that (*i*) there are no strict sequence requirements for protein-primed initiation on single-stranded DNA; (*ii*) initiation of replication occurs opposite the second nucleotide at the 3′ end of the template; (*iii*) a terminal repetition of at least two nucleotides is required to efficiently elongate the initiation complex; and (*iv*) all the nucleotides of the template, including the 3′ terminal one, are replicated. A sliding-back model is proposed in which a special transition step from initiation to elongation can account for these results. The possible implication of this mechanism for the fidelity of the initiation reaction is discussed. Since all the terminal protein-containing genomes have some sequence reiteration at the DNA ends, this proposed sliding-back model could be extrapolable to other systems that use proteins as primers.**

One of the mechanisms to initiate linear DNA replication makes use of specific proteins (terminal proteins, TPs), covalently linked to the 5′ DNA ends, that provide the hydroxyl group required to initiate DNA synthesis. In this mechanism, a specific DNA polymerase catalyzes the covalent linkage of the initiating nucleotide to the hydroxyl group of a serine, threonine, or tyrosine residue of the primer TP (reviewed in ref. 1).

Bacteriophage ϕ29 of *Bacillus subtilis* has a linear double-stranded DNA (dsDNA) genome, 19,285 base pairs (bp) long (2), with a 6-bp inverted terminal repeat (3′-TTTCAT-5′; refs. 3 and 4) and a TP covalently linked to each 5′ end (5). To initiate replication, the viral protein p6 forms a nucleoprotein complex at the viral replication origins, located at the DNA ends, and produces a conformational change in the DNA (6) that probably induces the unwinding of the double helix to expose a region of single-stranded DNA (ssDNA). A complex formed between the ϕ29 DNA polymerase and a free molecule of the TP (7) recognizes the replication origins and initiates replication through formation of a covalent bond between dAMP and the hydroxyl group of Ser^{232} of the TP (8, 9). This reaction requires divalent metal cations, Mn^{2+} being the best activator (10). After this initiation step, the same DNA polymerase catalyzes chain elongation by a strand-displacement mechanism (11, 12).

The DNA sequence requirements for the initiation reaction were first studied by cloning terminal fragments from both ϕ29 DNA ends. Restriction fragments having the ϕ29 terminal sequences at one of the DNA ends were active templates for the initiation reaction (13). Analysis of deletion derivatives indicated that the minimal replication origins were the terminal 12 bp at each ϕ29 DNA end (14). Point mutagenesis in those sequences showed the existence of some sequence requirements for template activity. In this paper we show that a variety of ssDNA molecules are able to support protein-primed initiation and elongation by ϕ29 DNA polymerase. Using synthetic oligonucleotides, we carried out a mutational analysis of the ϕ29 right replication origin to find out whether a specific ssDNA sequence was required for effective protein-primed initiation. This approach also allowed us to determine which of the three consecutive T residues at the 3′ ends was directing the first dAMP to the TP. The results indicate that there are no strict sequence requirements in ssDNA and that the initiation site for protein priming is the second nucleotide at the 3′ end of the ϕ29 DNA template strand. The effect of the mutations on the efficiency of replication and a model to account for the recovery of the terminal nucleotide are discussed.

MATERIALS AND METHODS

Nucleotides, Proteins, and Templates. Unlabeled deoxynucleotides and dideoxynucleotides were purchased from Pharmacia P-L Biochemicals. The four [α-^{32}P]dNTPs (410 Ci/mmol; 1 Ci = 37 GBq) were from Amersham International. The wild-type and exonuclease-deficient (D12A/D66A; ref. 15) ϕ29 DNA polymerases were purified essentially as described (8), with modifications to be published elsewhere. ϕ29 TP and protein p6 were purified as described (16, 17).

ϕ29 DNA-TP complex (TP-DNA) was isolated as described (18). oriL(77) and oriL(77)T2G (containing the terminal 77 bp of the ϕ29 DNA left replication origin, or the same sequence with a T → G transversion in the second nucleotide from the 3′ end, respectively) are restriction fragments of recombinant DNAs G7 and R161, respectively (14), digested with either *Dra* I or *Dra* I/*Rsa* I [oriL(77)] and *Rsa* I [oriL(77)T2G]. Circular ssDNA from mID13, a derivative of phage M13mp8, contains sequences (77 bases and 125 bases) of both ϕ29 replication origins. Poly(dC) and poly(dT) were from Pharmacia P-L Biochemicals. Single-stranded oligonucleotides were synthesized in a 391 DNA synthesizer (Applied Biosystems) (19). Oligonucleotides with the se-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TP, terminal protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

†To whom reprint requests should be addressed.

quence of the template strand of the ϕ29 DNA right origin are named oriR(n)t, where n is the length in nucleotides. Oligonucleotides with the sequence of the displaced strand are named oriR(n)d. Point substitutions in the oriR(12)t sequence are indicated by a three-symbol code: the first letter is the nucleotide to be changed; the number indicates its position from the 3′ end; the second letter is the nucleotide introduced (e.g., T1A is the oligonucleotide in which the first T has been changed to A). When several changes are introduced simultaneously, all of them are specified (e.g., T1C/T2C/T3C). The oligonucleotide with one T inserted in the 3′ end is named 3′VT. Oligonucleotides with one or two T residues deleted from the 3′ end are named T1Δ and T1Δ/T2Δ, respectively. The sequence of oriR(29)t is 5′-TGGTGTATGTTGTCGCTGTACCCTACTTT-3′.

TP-dNMP Initiation Complex Formation. The standard incubation mixture (25 μl) contained 50 mM Tris·HCl (pH 7.5), 1 mM dithiothreitol, 4% (vol/vol) glycerol, 1 mM $MnCl_2$, 20 mM ammonium sulfate, 2.5 μg of bovine serum albumin, 6.25 pmol of the [α-^{32}P]dNTP used, 125 ng of both ϕ29 TP and exonuclease-deficient ϕ29 DNA polymerase, and the indicated amount and type of template. When indicated, 10 mM $MgCl_2$ was added instead of $MnCl_2$, wild-type ϕ29 DNA polymerase was used instead of exonuclease-deficient ϕ29 DNA polymerase, and protein p6 (3 μg) was added. After incubation as indicated, the reaction was stopped by adding EDTA (10 mM) and SDS (0.1%). The samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS to remove unincorporated [α-^{32}P]dNTP and analyzed by SDS/PAGE (11). Quantitation was done by excising from the gel the band corresponding to the TP-dNMP complex and counting the Cerenkov radiation or by densitometry of the autoradiographs in a 300A computing densitometer (Molecular Dynamics).

TP-Primed DNA Replication. The replication assay was performed in similar conditions as the initiation assay, but with the four unlabeled dNTPs in addition to the corresponding initiating [α-^{32}P]dNTP. After incubation at 30°C, reactions were stopped and analyzed as described for the initiation assay. Percent elongation is given by the ratio between the radioactivity present in the elongation band and total radioactivity (initiation plus elongation bands). A correction is made taking into account the [α-^{32}P]dNTP used and the sequence of each oligonucleotide. ddTTP-truncated replication assays were carried out, essentially as described above, but in this case only the dNTPs required to reach the first A in the template were added (20 μM dATP and 20 μM dGTP). In all cases, 200 μM ddTTP was present. To obtain resolution enough to distinguish the TP bound to the various elongation products, the samples were analyzed in SDS/12% polyacrylamide gels (360 × 280 × 0.5 mm).

RESULTS

ssDNA Can Support TP-Primed Initiation. The possibility that replication of ssDNA could be initiated by a protein-priming mechanism was tested with both heat-denatured dsDNA and synthetic oligonucleotides. A heat-denatured linear DNA molecule containing 77 bases of the ϕ29 left origin [oriL(77)ss] at one end was able to support the formation of the TP-dAMP initiation complex to an extent similar to or even higher (depending on the temperature) than that obtained with the same molecule in native form [oriL(77)ds] (Fig. 1). A synthetic single-stranded oligonucleotide 29 bases long, with the sequence corresponding to the ϕ29 right origin template-strand [oriR(29)t] was also active in the *in vitro* initiation reaction. However, a covalently closed circular ssDNA containing ϕ29 DNA terminal sequences (mID13) was inactive (Fig. 1), indicating that the DNA polymerase/TP complex requires a free ssDNA end, as was the case with

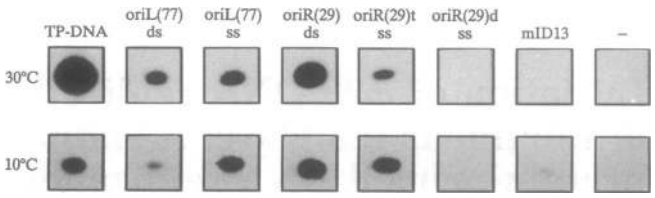

FIG. 1. Formation of the TP-dAMP initiation complex with ds- and ssDNA templates. Reactions were carried out for 5 min at 30°C and 10°C with various templates: TP-DNA, 500 ng; oriL(77)ds, a 2063-bp *Dra* I restriction fragment containing 77 bp of ϕ29 left replication origin, 214 ng; oriL(77)ss, obtained by heat-denaturation of oriL(77)ds, 214 ng; oligonucleotides oriR(29)t and oriR(29)d, 75 ng; oriR(29), obtained by hybridization of oriR(29)t and oriR(29)d, 150 ng; ssDNA from mID13, containing sequences (77 bases and 125 bases) of both ϕ29 DNA ends, 200 ng. A control in the absence of DNA (–) was carried out. The formation of the TP-dAMP initiation complex was analyzed by SDS/PAGE and autoradiography. Only the area containing the relevant band is shown.

dsDNA (13). oriR(29)d, the oligonucleotide complementary to oriR(29)t, was inactive in the formation of the TP-dAMP initiation complex. As a control, oriR(29)ds, the hybrid formed by oriR(29)t and oriR(29)d, was an efficient template for initiation. The reactions with the natural ϕ29 template (TP-DNA) or in the absence of any template are also shown in Fig. 1. When the initiation assay was carried out at 10°C, the efficiency of the reaction was decreased relative to that at 30°C with dsDNA as template but was not affected or was increased with ssDNA.

To ascertain whether the initiation reaction obtained with ssDNA molecules was actually directed by a complementary nucleotide (T), homopolymers were used as initiation templates with each of the four [α-^{32}P]dNTPs as substrate. With poly(dT) as template, dATP was the preferred nucleotide to be linked to the TP, whereas with poly(dC), dGTP was the one preferentially used (data not shown). These results indicate that protein-primed initiation is template-directed.

TP-Primed Initiation Is Directed by the Second Nucleotide at the 3′ End of the Template Strand. Mutational analysis of the ϕ29 3′ right end was carried out with single-stranded dodecanucleotides. Single changes were introduced in the first, second, or third position from the 3′ end of oligonucleotide oriR(12)t (oligonucleotides T1A, T1C, T1G, T2A, T2C, T2G, and T3G; for nomenclature see *Materials and Methods*). These oligonucleotides were assayed for TP-primed initiation with each of the four [α-^{32}P]dNTPs as substrate. Alteration of the first or the third position did not produce any change in the specificity for the nucleotide, [α-^{32}P]dATP being the one preferentially incorporated (Fig. 2 and data not shown). On the other hand, changes in the second position produced a drastic effect; reactions with [α-^{32}P]dATP were rather low, and the nucleotide complementary to the second position at the 3′ end of the template was the one preferentially bound to the TP (Fig. 2), strongly suggesting that the initiation reaction with these ssDNA templates was directed by the second nucleotide from the 3′ end. These results were confirmed by using different oligonucleotides of either unrelated sequences or with new changes introduced in the right origin sequence. In all cases a preference for the [α-^{32}P]dNTP complementary to the second nucleotide at the 3′ end of the template was observed (data not shown).

Two heat-denatured DNA molecules, 1201 bp long, obtained as restriction fragments from recombinant DNAs, containing 77 bases of the ϕ29 left origin [oriL(77)] or the same sequence with a T → G change in the second position [oriL(77)T2G], were tested in the initiation reaction with each of the four [α-^{32}P]dNTPs as substrate. The results (Fig. 2) indicated again that initiation was mainly directed by the second nucleotide at the 3′ end of the template. When

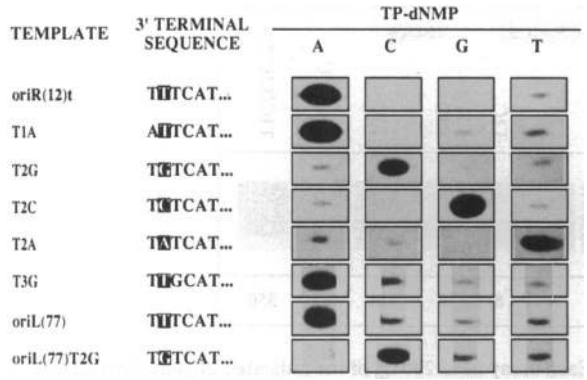

FIG. 2. Protein-primed initiation with each of the four dNTPs, using ssDNA templates with altered sequences at the 3′ end. The formation of the four TP-dNMP complexes was assayed and analyzed. The 3′ terminal sequence of each template is indicated. Incubation was for 5 min at either 30°C [oriL(77) and oriL(77)T2G templates] or 10°C (other templates). Templates were oligonucleotides oriR(12)t, T1A, T2G, T2C, T2A, and T3G, 200 ng; oriL(77) (heat-denatured *Dra* I–*Rsa* I restriction fragment, 1201 bp); and oriL(77)T2G (heat-denatured *Rsa* I restriction fragment, 1201 bp), 20 ng. The efficiency of the reaction obtained with the preferred nucleotide in each case was as follows: oriR(12)t, 100%; T1A, 110%; T2A, 96%; T2C, 140%; T2G, 69%; T3G, 73%. In oriL(77) and oriL(77)T2G, the sequence corresponding to the 3′ end of the displaced strand (3′-TGCGCG-5′) was probably responsible for some of the initiation reaction obtained with dCTP.

fragments oriL(77) and oriL(77)T2G were assayed in native form, in the presence of the wild-type ϕ29 DNA polymerase and $MgCl_2$, initiation also took place opposite the second nucleotide at the 3′ end (results not shown), thus indicating that this phenomenon was neither exclusive for ssDNA templates nor due to the DNA polymerase or the metal cation used. Moreover, when these dsDNA fragments were assayed in the presence of the origin-specific DNA-binding protein p6, the initiation reaction, stimulated 3- to 5-fold, was again directed by the second nucleotide at the 3′ end of the template (data not shown).

TP-Primed Replication of Single-Stranded Oligonucleotides: Recovery of the First 3′ Nucleotide. With the demonstration that initiation was directed by the second nucleotide from the 3′ end, the next question was whether the TP-dNMP initiation complex formed could be elongated by the ϕ29 DNA polymerase; if this occurred, it would be necessary to investigate whether the replication product had the same length as the template oligonucleotide or whether one base (the one complementary to the 3′ end of the template) is lacking. Oligonucleotide oriR(29)t was assayed for replication using different concentrations of dNTPs. PAGE analysis showed two bands: one corresponded to the initiation complex and the other one, which increased with the concentration of dNTPs, to the elongated product (data not shown).

To determine whether the nucleotide complementary to the first position of the template was recovered during elongation, a replication assay was carried out in the presence of ddTTP, allowing elongation to take place only to the first A of the template. This experiment was done with the natural ϕ29 template (TP-DNA), as a control, and oligonucleotides oriR(12)t, 3′∇T, and T1Δ. Special conditions for gel electrophoresis were used (see *Materials and Methods*) to resolve the various TP-$(dNMP)_n$ elongation products. When TP-DNA or oriR(12)t was used as template, five bands were observed (Fig. 3), indicating that the longest product was TP-AAAGT*. Therefore, despite the fact that initiation occurred at the second nucleotide, all the positions of the template, including the first nucleotide, seemed to be replicated. Furthermore, a longer product, probably corresponding to TP bound to a hexanucleotide, appeared when 3′∇T (having an additional T at the 3′ end) was used; when the first T at the 3′ end was deleted (T1Δ), the longest product corresponded to TP bound to a tetranucleotide. A possible mechanism to account for these results will be discussed later.

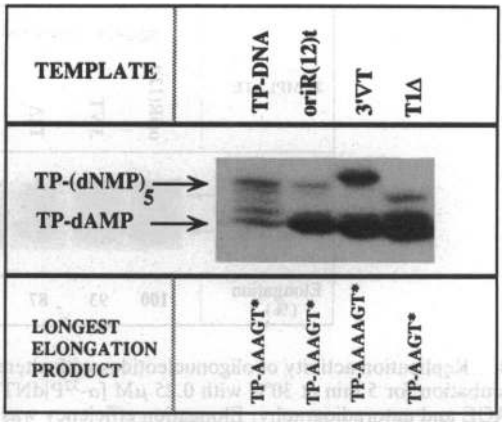

FIG. 3. Truncated replication assay. The assay was carried out with the indicated template: TP-DNA, 500 ng; oligonucleotides oriR(12)t, 3′∇T, and T1Δ, 200 ng. After 5 min at 30°C in the presence of 20 μM [α-^{32}P]dATP (2.5 μCi), 20 μM dGTP, and 200 μM ddTTP, the products obtained were analyzed by SDS/PAGE. The longest product expected in each case is indicated. T*, ddTMP incorporated to stop elongation.

Requirement of a Terminal Repetition to Elongate the TP-dNMP Initiation Complex. To determine whether particular sequences at the 3′ end of the template were important for elongation of the TP-dNMP complex, various dodecanucleotides were assayed for TP-primed replication. In each case, the [α-^{32}P]dNTP complementary to the second position of the template was supplied as initiating nucleotide. In the presence of 20 μM dNTPs, the oligonucleotide oriR(12)t was replicated (Fig. 4). Templates with substitutions in positions T1 (T1A, T1C, and T1G) and T2 (T2A, T2C, and T2G) were strongly affected in their replication capability (values ranged from 0 to 24% of the one corresponding to the wild-type sequence). Conversely, insertion or deletion of one T, or the change of the third T to G (T3G), did not produce such a negative effect (values 51–93% of the wild type). Interestingly, when the two terminal T residues were changed to C residues (T1C/T2C), the oligonucleotide could support replication with an efficiency 63% of that obtained with the wild-type sequence, and the substitution of the three T residues (T1C/T2C/T3C) in the same oligonucleotide led to an increased replication activity (350%) (Fig. 4). These results suggest that a repetition of at least two nucleotides at the DNA end is required for efficient elongation of the TP-dNMP initiation complex.

DISCUSSION

It is generally accepted that initiation of DNA replication involves at least three differentiated steps: (*i*) recognition of the replication origin; (*ii*) unwinding of the double helix to expose a ssDNA region; (*iii*) synthesis or positioning of a priming molecule. Using an *in vitro* system that replicates ϕ29 DNA-TP with the ϕ29 DNA polymerase and the TP as the only proteins (11), we show that dsDNA is not an essential requirement for protein-primed ϕ29 DNA replication, since linear ssDNA molecules are active templates for initiation and can also undergo replication by the DNA polymerase.

We have prepared oligonucleotides with the sequence of the ϕ29 DNA right origin of replication, as well as altered sequences with single or multiple changes in the first three

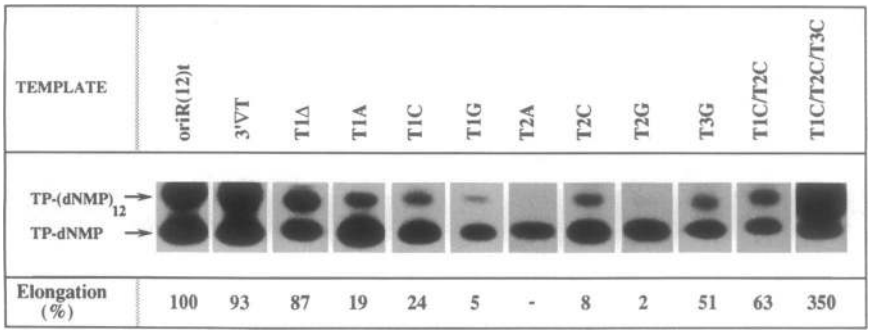

FIG. 4. Replication activity of oligonucleotides with altered 3′ sequences. Each assay used 200 ng of the indicated oligonucleotide template. After incubation for 5 min at 30°C with 0.25 μM [α-^{32}P]dNTP (initiating nucleotide) and 20 μM unlabeled dNTPs, samples were analyzed by SDS/PAGE and autoradiography. Elongation efficiency was determined by the ratio of elongation [TP-(dNMP)$_{12}$] to initiation plus elongation [TP-dNMP plus TP-(dNMP)$_{12}$]. Value for oriR(12)t was taken as 100%.

nucleotides at the 3′ end. When these oligonucleotides were used as templates for initiation, the absolute values obtained indicated no strict requirement for a specific sequence in the DNA. This lack of specificity with ssDNA templates has been described in adenovirus (20, 21), although other reports (22, 23) suggest that certain specificity of sequence in the adenovirus origin is required on ssDNA for the *in vitro* initiation of replication. However, this apparent specificity could be explained as a requirement for a precise nucleotide in the right position of the template to direct initiation (see below).

More interestingly, the results obtained using single-stranded oligonucleotides with altered sequences in the first three nucleotides at the 3′ end indicated that, in φ29, the initiation reaction is directed by the second nucleotide from the 3′ end of the DNA. To further check this unexpected result, we have also used dsDNA molecules as templates, in the presence of the activating protein p6, which forms a nucleoprotein complex at the φ29 DNA origins (6), to mimic as much as possible the natural conditions for initiation of φ29 DNA replication. Using a DNA containing 77 bp of the φ29 left origin of replication with a T → G transversion in the second nucleotide at the 3′ end, initiation took place opposite the second position, both in the absence and in the presence of protein p6.

Although no specific sequences were required for the initiation reaction, the presence of a 3′ terminal sequence of at least two T residues in the template was required for efficient elongation. Alterations of this repetition (as in oligonucleotides with changes in T1 or T2) strongly diminished the replication capability, whereas an oligonucleotide with both changes in the sequence (T1C/T2C) was replicated efficiently. All the nucleotides of the template, even the 3′ terminal one, seem to be replicated. Since initiation in φ29 is directed by the second nucleotide of the template, probably a special first translocation step is needed to recover the terminal nucleotide. This is in agreement with the fact that elongation of the TP-dAMP complex is an especially difficult step in φ29 DNA replication (24). Therefore, we propose a sliding-back mechanism for the transition from initiation to elongation that could account for the necessity of a terminal repetition for efficient elongation and also for the recovery of the information corresponding to the 3′ end of the template. As shown in Fig. 5, the second nucleotide of the template (T) directs the linkage of dAMP to Ser^{232} of the TP (initiation). Then, this TP-dAMP initiation complex slides backwards (transition), locating dAMP in front of the first nucleotide of the template (T). In this transition step, which probably leads to the dissociation of the DNA polymerase/TP heterodimer, the following nucleotide (A) is incorporated, using again the second T of the template as a director. Further DNA synthesis (elongation) involves a normal translocation of the enzyme along the template. This mechanism could explain the low replication activity obtained with the oligonucleotides with changes in T1 or T2. In both cases, the initiation reaction occurs with the dNTP complementary to the second base at the 3′ end of the DNA. Nevertheless, after the transition step, the first nucleotide incorporated is located in front of the terminal nucleotide of the template, producing an incorrect base pairing. This mismatch would produce dissociation of initiation complexes, which would not be elongated.

Genomes that contain a TP have some sequence repetition at the DNA ends (Fig. 6). This suggests that the sliding-back mechanism proposed for φ29 DNA replication could be extrapolated to other systems that use proteins as primers. In fact, the mutational analysis carried out in the adenovirus type 2 replication origin, whose terminal reiteration is 3′-GTAGTA-5′, revealed that the oligonucleotide with the first G changed to an A was fully active in initiation with dCTP, while the double substitution of the first and fourth G residues

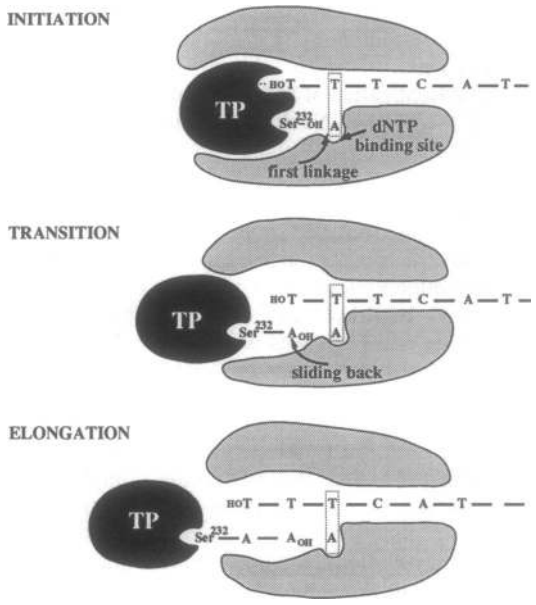

FIG. 5. Sliding-back model for transition from initiation to elongation. Protein-primed initiation occurs opposite the second 3′ nucleotide of the template (initiation). The initiation complex (TP-dAMP) slides back, setting the initiating dAMP base-paired to the 3′ terminal nucleotide of the template (transition). The translocated initiation complex is elongated to fully replicate the template (elongation). The 3′ hydroxyl group of the template is tentatively depicted interacting with TP. Light shaded areas correspond to the polymerization domain of φ29 DNA polymerase, defining a cleft proposed to be used as both DNA and TP binding sites.

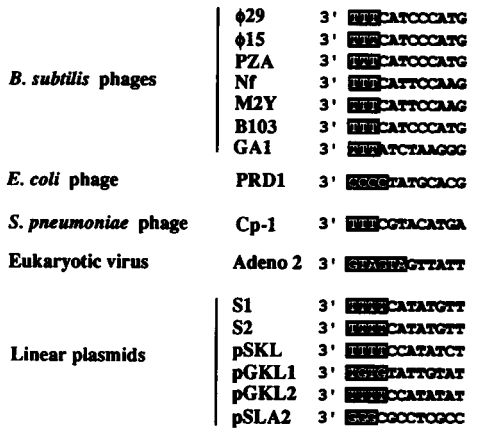

FIG. 6. The 3′ terminal nucleotide sequences of TP-containing DNA genomes. Sequence references are reviewed in ref. 1; terminal iteration is indicated in white letters. *E. coli, Escherichia coli; S. pneumoniae, Streptococcus pneumoniae*; Adeno, adenovirus.

completely inactivated the template (23). This result could be explained if initiation takes place at the G at the fourth position of the template. It has been proposed that this reiteration (GTAGTA) would allow regeneration of missing terminal sequences in adenovirus type 5 by a strand-slippage mechanism (25). A mutational analysis made in the replication origin of bacteriophage PRD1, which contains four C residues at the 3′ end of the DNA, revealed that changes in the first, second, or third terminal nucleotides did not abolish the initiation reaction, whereas change of the fourth C to A made the oligonucleotide inactive in initiation with dGTP (26). These results are consistent with an internal initiation, probably at the fourth C residue from the 3′ end.

In a number of RNA viruses, such as bacteriophage Qβ (27, 28) and brome mosaic virus (29), initiation of the (−)-strand synthesis occurs opposite the second 3′ nucleotide of the template. In addition, in Semliki forest virus (30), Sindbis virus (31), cucumber mosaic virus (32), and brome mosaic virus (29), replicative-form RNA contains an unpaired G at the 3′ end of the (−)-strand, suggesting that (+)-strand initiation may also take place opposite the second 3′ nucleotide. In certain of these RNA viruses, the fact that the 5′ end of the (−)-strand is not blocked by the presence of a TP allows the recovery of the first position of the template by means of a postreplicative addition event (29, 33). Interestingly, it has been recently suggested that initiation of Tacaribe arenavirus RNA replication occurs at the second position and that the initiation complex slips backwards before elongation can continue (34). This finding parallels, in RNA replication, the results presented in this paper for initiation of ϕ29 DNA replication.

The fidelity of the protein-primed initiation step (TP-dAMP complex formation) of ϕ29 DNA replication is lower than that of DNA polymerization steps (36). Therefore, it should be important to have a mechanism to increase the fidelity during the initiation reaction. According to the proposed sliding-back mechanism (see Fig. 5), in the transition step, the nucleotide linked to the TP will be located opposite the first base of the template. Taking into account that the first (T) and second (T) template nucleotides are identical, if a mismatched initiation occurs, transition to elongation would not be efficient, and the incorrect initiation complex would dissociate from the DNA. The concomitant removal of both TP and wrong nucleotide may be the only possible way for editing to occur, because the exonucleolytic activity of ϕ29 DNA polymerase, which acts as a proofreading enzyme during elongation (35), cannot excise the first dNMP linked to the TP (36). Nonetheless, if a mutation is established in one of the two first nucleotides of the DNA, the mutated molecule will not be an efficient template for the next round of replication. The quantitative contribution of this nonexonucleolytic proofreading mechanism to the global fidelity of protein-primed initiation remains to be elucidated.

We are grateful to J. A. García for helpful discussions and to J. M. Lázaro for enzyme purification. This investigation has been aided by Research Grant 5R01 GM27242-12 from the National Institutes of Health, by Grant PB90-0091 from Dirección General de Investigación Científica y Técnica, by Grant BIOT CT91-0268 from the European Economic Community, and by an institutional grant from Fundación Ramón Areces. J.M. and J.A.E. were predoctoral fellows from Ministerio de Educación y Ciencia, and A.B. was a postdoctoral fellow from Consejo Superior de Investigaciones Científicas.

1. Salas, M. (1991) *Annu. Rev. Biochem.* **60**, 39–71.
2. Vlček, C. & Pačes, V. (1986) *Gene* **46**, 215–225.
3. Escarmís, C. & Salas, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1446–1450.
4. Yoshikawa, H., Friedman, T. & Ito, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1336–1340.
5. Salas, M., Mellado, R. P., Viñuela, E. & Sogo, J. M. (1978) *J. Mol. Biol.* **119**, 269–291.
6. Serrano, M., Salas, M. & Hermoso, J. M. (1990) *Science* **248**, 1012–1016.
7. Blanco, L., Prieto, I., Gutiérrez, J., Bernad, A., Lázaro, J. M., Hermoso, J. M. & Salas, M. (1987) *J. Virol.* **61**, 3983–3991.
8. Blanco, L. & Salas, M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5325–5329.
9. Hermoso, J. M., Méndez, E., Soriano, F. & Salas, M. (1985) *Nucleic Acids Res.* **13**, 7715–7728.
10. Esteban, J. A., Bernad, A., Salas, M. & Blanco, L. (1992) *Biochemistry* **31**, 350–359.
11. Blanco, L. & Salas, M. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6404–6408.
12. Blanco, L., Bernad, A., Lázaro, J. M., Martín, G., Garmendia, C. & Salas, M. (1989) *J. Biol. Chem.* **264**, 8935–8940.
13. Gutiérrez, J., García, J. A., Blanco, L. & Salas, M. (1986) *Gene* **43**, 1–11.
14. Gutiérrez, J., Garmendia, C. & Salas, M. (1988) *Nucleic Acids Res.* **16**, 5895–5914.
15. Bernad, A., Blanco, L., Lázaro, J. M., Martín, G. & Salas, M. (1989) *Cell* **59**, 219–228.
16. Zaballos, A. & Salas, M. (1989) *Nucleic Acids Res.* **17**, 10353–10366.
17. Pastrana, R., Lázaro, J. M., Blanco, L., García, J. A., Méndez, E. & Salas, M. (1985) *Nucleic Acids Res.* **13**, 3083–3100.
18. Peñalva, M. A. & Salas, M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5522–5526.
19. Strauss, F. C., Lobori, J. A., Siu, G. & Hood, L. E. (1986) *Anal. Biochem.* **154**, 353–360.
20. Challberg, M. D. & Rawlins, D. R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 100–104.
21. Guggenheimer, R. A., Stillman, B. W., Nagata, K., Tamanoi, F. & Hurwitz, J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3069–3073.
22. Harris, M. P. G. & Hay, R. T. (1988) *J. Mol. Biol.* **201**, 57–67.
23. Dobbs, L., Zhao, L. J., Sripad, G. & Padmanabhan, R. (1990) *Virology* **178**, 43–51.
24. Blanco, L., Gutiérrez, J., Lázaro, J. M., Bernad, A. & Salas, M. (1986) *Nucleic Acids Res.* **14**, 4923–4937.
25. Graham, F. L., Rudy, J. & Brikley, P. (1989) *EMBO J.* **8**, 2077–2085.
26. Yoo, S. K. & Ito, J. (1991) *J. Mol. Biol.* **218**, 779–789.
27. Rensing, U. & August, J. T. (1969) *Nature (London)* **224**, 853–856.
28. Goodman, H. M., Billeter, M. A., Hindley, J. & Weissmann, C. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 921–928.
29. Miller, W. A., Bujarski, J. J., Dreher, T. W. & Hall, T. C. (1986) *J. Mol. Biol.* **187**, 537–546.
30. Wengler, G., Wengler, G. & Gross, H. J. (1979) *Nature (London)* **282**, 754–756.
31. Wengler, G., Wengler, G. & Gross, H. J. (1982) *Virology* **123**, 273–283.
32. Collmer, C. W. & Kaper, J. M. (1985) *Virology* **145**, 249–259.
33. Bausch, J. N., Kramer, F. R., Miele, E. A., Dobkin, C. & Mills, D. R. (1983) *J. Biol. Chem.* **258**, 1978–1984.
34. Garcin, D. & Kolakofsky, D. (1992) *J. Virol.* **66**, 1370–1376.
35. Garmendia, C., Bernad, A., Esteban, J. A., Blanco, L. & Salas, M. (1992) *J. Biol. Chem.* **267**, 2594–2599.
36. Esteban, J. A., Salas, M. & Blanco, L. *J. Biol. Chem.*, in press.