Specific Recognition of Parental Terminal Protein by DNA Polymerase for Initiation of Protein-primed DNA Replication*

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The linear genome of Bacillus subtilis phage φ29 has a protein covalently linked to the 5' ends, called parental terminal protein (TP), and is replicated using a free TP as primer. The initiation of phage φ29 DNA replication requires the formation of a DNA polymerase/TP complex that recognizes the replication origins located at the genome ends. The DNA polymerase catalyzes the formation of the initiation complex TP-dAMP, and elongation proceeds coupled to strand displacement. The same mechanism is used by the related phage Nf. However, DNA polymerase and TP from φ29 do not initiate the replication of Nf TP-DNA. To address the question of the specificity of origin recognition, we took advantage of the initiation reaction enhancement in the presence of Mn2⁺, allowing us to detect initiation activity in heterologous systems in which DNA polymerase, TP, and template TP-DNA are not from the same phage. Initiation was selectively stimulated when DNA polymerase and TP-DNA were from the same phage, strongly suggesting that specific recognition of origins is brought through an interaction between DNA polymerase and parental TP.

The process of initiation of DNA replication implies, prior to nascent DNA synthesis, recognition of origins, unwinding of dsDNA, and priming. These universal events require various DNA-protein and protein-protein interactions that widely differ among replicons (reviewed in Refs. 1–3). One of the simplest models for origin recognition and initiation of replication has been proposed for linear dsDNA genomes with a covalently attached TP. Free TP acts as a primer (primer TP) for DNA replication, remaining linked to the 5' ends of the fully replicated molecule (parental TP) to constitute the replication origins. TP-DNAs have been found in bacteriophages (e.g. φ29, Nf, GA-1, Cp-1, and PRD1), animal viruses (e.g. adenoviruses), plasmids (e.g. S1, Kalilo), and bacteria (e.g. Streptomyces) (reviewed in Refs. 4 and 5).

Bacillus subtilis phage φ29 initiates replication of its 19,285-bp-long linear DNA by a protein-priming mechanism that has been extensively studied. Phage-encoded DNA polymerase and primer TP form a complex (polTP) that recognizes the ends of TP-DNA. Then DNA polymerase catalyzes the covalent linkage of dAMP to the OH group of Ser232 of the TP, giving rise to the TP-DAMP initiation complex (reviewed in Ref. 4). The incorporation of the dAMP is directed by the Thy at the second position of the template strand, and full-length sequence is obtained by a sliding-back mechanism that aligns the Ade with the 3'-terminal Thy of the template (6). A similar mechanism has been described for the φ29-related phage GA-1 (7), the Escherichia coli phage PRD1 (8), and adenovirus (9). φ29 DNA polymerase elongates the initiation complex by a strand displacement mechanism in a very processive way, with no further requirements for any helicase or processivity factors (reviewed in Ref. 10). In addition to DNA polymerase and TP, phage-encoded ss- and dsDNA-binding proteins (p5 and p6, respectively) are required for φ29 DNA replication in vitro. Protein p5 stimulates replication in vitro (11) by interacting with the displaced single-stranded DNA (12). Protein p6, which has been proposed to play a role organizing and compacting the viral genome (13), activates the initiation of replication by forming multimeric nucleoprotein complexes at the ends of φ29 DNA (14, 15). No auxiliary host proteins have been described to be required for φ29 DNA replication, unlike the case of adenoviruses, which also replicate by a protein-priming mechanism (reviewed in Refs. 16 and 17), where two cellular transcription factors, NFI and Oct-1, have been reported to interact with DNA polymerase and pTP (φ29 TP counterpart), respectively, directing the multiprotein complexes to the viral origins of replication (18–20).

Synthesis of the φ29 TP-dAMP complex in vitro requires the formation of a stable, equimolar, polTP complex (21) that recognizes the replication origins at the genome ends. The main signal to be recognized by the polTP complex for initiation of replication is the parental TP. Thus, when terminal DNA fragments lacking parental TP are used as templates, the initiation reaction becomes much less efficient, being about 10% of the activity obtained with TP-DNA (Ref. 22 and this work). Even in the absence of any template, DNA polymerase does retain the ability to deoxynucleotidylate TP unspecifically, although at a very low rate (23).

φ29 belongs to a family of phages classified into three groups (reviewed in Ref. 24) as follows: group A comprises phages BS32, φ15, and PZA together with φ29; group B includes M2, Nf, and B103, and group C has GA-1 as its sole member. The DNA polymerases of φ29 and Nf are 572 amino acid residues long and share an 81.8% sequence identity (Ref. 25 and this work). The TP of φ29 and Nf have 266 amino acids with a 62.4% sequence identity (26, 27). φ29 TP complements in vivo PZA but not M2 TP sus mutants (28). Furthermore, in vitro

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1 The abbreviations used are: dsDNA, double-stranded DNA; TP, terminal protein; TP-DNA, TP covalently linked to DNA; polTP, DNA polymerase-TP complex; bp, base pair; BSA, bovine serum albumin; oriL, left terminal DNA fragment; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
assays showed that the φ29 pol/TP complex is able to initiate DNA replication using PZA or φ15 TP-DNAs as template but not NDTP-DNA (22), suggesting the need of a TP-DNA from the same phage group for replication. The use of Mn²⁺ instead of Mg²⁺ in the in vitro system, which increases the formation of the initiation complex (Ref. 29 and this work), allowed us to detect initiation activity with pol/TP proteins from φ29 or Nf and TP-DNAs from Nf or φ29, respectively. This result prompted us to study the specificity of the template recognition by the initiation proteins (pol/TP). For this, we purified DNA polymerase and TP from the phage Nf to assay initiation in φ29/Nf heterologous replication systems.

In this paper, we present evidence that the initiation activity is higher when the DNA polymerase is from the same phage as the TP-DNA, suggesting that a specific interaction between DNA polymerase and parental TP is essential for efficient initiation of replication and determines the specificity of origin recognition.

MATERIALS AND METHODS

Nucleotides, Oligonucleotides, and Enzymes—U-labeled nucleotides and [γ-²³⁰P]dATP (3,000 Ci/mmol), as well as micrococcus nuclease were purchased from Amersham Pharmacia Biotech. Oligonucleotides were obtained from Boehringer Ingelheim and isogen. 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. Sequenase 2.0 version of T7 DNA polymerase was from United States Biochemical Corp.

DNA Templates—φ29 and Nf TP-DNAs (30) and proteinase K-digested φ29 and Nf DNAs (31) were obtained as described. φ29 and Nf DNA left terminal fragments (orL), 259 and 263 bp, respectively, were obtained by PCR amplification from proteinase K-digested DNAs. Oligonucleotides were designed to produce a Dral restriction site to regenerate the genome end. In the case of φ29, the PCR amplification product was phosphorylated and cloned in a Smal-digested and dephosphorylated pBlueScript cloning vector, giving rise to p259. E. coli DH5α cells were transformed and plated on LB medium containing 100 µg/ml ampicillin. Selection of p259-containing colonies was carried out by assessing β-galactosidase activity. Transformants were grown in selective medium at 37°C, and φ29 orL was obtained after restriction of purified p259 with Dral and EcoRV. This digestion results in the 259-terminal bp from φ29 plus 18 additional bp from the plasmid. DNA fragments were purified from agarose gels using the Qia Quick Gel Extraction Kit of Qiagen.

Cloning, Expression, and Purification of φ29 and Nf DNA Polymerases—φ29 DNA polymerase was obtained from E. coli NF2900 cells harboring plasmid pJLw2 and purified as described (32). Nf DNA polymerase gene was cloned and overproduced in E. coli cells. For this, phage Nf was obtained from infected B. subtilis cells and the NDNA2690 cells were grown at 37°C in LB medium up to an optical density of 0.5 at 600 nm infected with phage Nf at a multiplicity of 10. Cells were harvested 50 min after infection, and cell-free extracts were obtained as described above. After ammonium sulfate precipitation, TP was purified by phosophocellulose and calf thymus DNA-cellulose chromatography eluting at 0.5 M and 1 mM NaCl in buffer 6, respectively. The protein was further purified through a heparin-agarose column, eluting at 1 mM NaCl in buffer 6. The sample was concentrated to a Centricon-30. The protein was over 95% homogeneous as estimated by SDS-PAGE and Coomassie Blue staining.

DNA Polymerase-TP Interaction—Interaction between DNA polymerase (25 µg) and TP (12.5 µg) from φ29 and Nf was analyzed by glycerol gradient centrifugation. The proteins, in 50 mM Tris-HCl, pH 7.5, 20 mM ammonium sulfate, 7 mM β-mercaptoethanol, 1 mM EDTA, 2 mM ZnSO4, 100 mM NaCl, and 8.5% glycerol were loaded a 4-ml 15–30% glycerol gradient together with 25 µg of BSA as molecular weight marker and centrifuged at 350,000 × g for 24 h at 4°C. After centrifugation, 0.16-ml fractions were collected, and aliquots were analyzed on SDS-PAGE. Quantification was performed by densitometric scans of Coomassie-stained gels in a Molecular Dynamics 300A densitometer.

Interaction between DNA polymerase and TP from φ29 and Nf was also analyzed by a DNA competition assay in which the 3′ filling-in of a DNA fragment is inversely proportional to the amount of DNA polymerase interacting with TP. A dsDNA fragment with a 3′-recessive end, substrate for DNA polymerase, was obtained by restriction of phi29 with SpeI and PstI. DNA polymerase and TP from φ29 and Nf were preincubated for 50 min at 4°C. The DNA fragment was added to a reaction mixture containing, in a 25-µl final volume, 50 mM Tris-HCl, pH 7.5, 20 mM ammonium sulfate, 5% glycerol, 5% polyethylene glycol 1000, 1 mM diithiothreitol, 0.1 mg/ml BSA, 32 mM NaCl, 1 mM MgCl₂, 33 nM [γ-²³⁰P]dATP (2.5 µCi), and 400 nM of each dCTP, dTTP, and dGTP. The reaction was carried out for 2 min at 30°C and stopped by addition of 10 µM EDTA and 0.1% SDS. Samples were analyzed by SDS-PAGE.

Assay for Protein-primed Initiation of DNA Replication—The reaction mixture, in 25 µl, was as described above except that the only deoxynucleotide present was 250 nM [γ-²³⁰P]dATP (2.5 µCi); 1 mM MnCl₂ and the indicated amounts of purified DNA polymerase, TP, and template DNA were used. The samples were incubated at 30°C for the indicated times, and the reaction was stopped by addition of EDTA up to 10 mM. Samples were heated at 60°C for 5 min, and 25 units of micrococcus nuclease for 30 min at 37°C in 50 mM Tris-HCl, pH 8.8, and 22 mM CaCl₂. The reaction was stopped as described above. Samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS and analyzed by SDS-PAGE. Quantification of the initiation complex formed was done by PhosphoImager densitometric scans of a exposed Fuji BAS-IIIs imaging plate.

RESULTS

Cloning and Sequencing of Nf DNA Polymerase Gene—To study the specificity of the protein-primed initiation of replication, we have used the in vitro systems of φ29 and the related phage Nf. Partially purified samples containing both DNA polymerase and TP from Nf were used in a previous work (36); however, the current goal required the availability of each of the proteins free of the other. Since it was difficult to obtain DNA polymerase free of contaminant TP from Nf-infected B. subtilis cells, we cloned the Nf DNA polymerase gene, after PCR amplification of the corresponding region. Since phage M2 is derived from phase Nf by a deletion that does not affect the replication genes (37), we took advantage of the known sequence of the M2 phage DNA polymerase gene (gene G) (38) for the design of oligonucleotides. Nf DNA polymerase predicted gene was sequenced, and the product was identical in size and amino acid sequence to that of M2 DNA polymerase (38), except for exon 107, where arginine was found instead of lysine. The predicted protein had a molecular mass of 66.4 kDa and 81.8% identity and 91.3% homology to its φ29 counterpart.

DNA Polymerase and TP from Phages φ29 and Nf Are Able to
Form Heterologous Complexes—Protein-primed initiation of DNA replication requires the prior formation of a complex between DNA polymerase and TP to recognize the replication origins (21). Studies on the specificity of the initiation reaction required the use of polTP complexes in which both proteins were from the same phage, \( \phi 29 \) or Nf (hereafter homocomplexes), or one from \( \phi 29 \) and the other from Nf (hereafter heterocomplexes). The formation of polTP complexes was analyzed by glycerol gradient centrifugation. TP (31 kDa) from either \( \phi 29 \) (Fig. 1A) or Nf (Fig. 1B) sedimented behind the 67-kDa molecular mass marker BSA that cosedimented with the \( \phi 29 \) and Nf DNA polymerases; however, when complexed with DNA polymerase, it sedimented slightly ahead of BSA, both in the case of \( \phi 29 \) (Fig. 1C) and Nf (Fig. 1D) homocomplexes. A similar shift was observed when \( \phi 29 \) and Nf heterocomplexes were analyzed (Fig. 1, E and F), although in the case of Nf DNA polymerase/\( \phi 29 \) TP about 40% of TP sedimented as free TP, suggesting they have a lower affinity (Fig. 1F); here, free and TP-complexed DNA polymerases are not resolved. Therefore, DNA polymerase and TP from the two phages are able to interact with each other although with different affinities. Nevertheless, when glycerol gradients were performed at 180 mM NaCl with a 10-fold lower amount of protein, polTP homo- but not heterocomplexes were detected (results not shown). This result indicates that DNA polymerase has a higher affinity for TP in homocomplexes than in heterocomplexes.

The interaction between the different DNA polymerases and TPs was further tested in an assay in which DNA polymerase binding to TP is challenged by a dsDNA fragment with a recessive 3′ end. Since TP binds to the same active site as a DNA primer in DNA polymerase (10) and they cannot bind simultaneously (39), the extent of the filling-in reaction would indicate the amount of free DNA polymerase, providing a qualitative measurement of the affinity of each DNA polymerase for each TP. The filling-in activities of both \( \phi 29 \) (Fig. 2, lane b) and Nf (Fig. 2, lane f) DNA polymerases were basically the same; therefore, their activities are comparable in these conditions. The DNA primer-template competed more efficiently for \( \phi 29 \) DNA polymerase when complexed with Nf TP (Fig. 2, lane e) than with \( \phi 29 \) TP (Fig. 2, lane d). Likewise, Nf DNA polymerase was more efficiently competed when complexed with \( \phi 29 \) TP (Fig. 2, lane i) than with NfTP (Fig. 2, lane h). These results fully agree with the previous observation of the higher affinity of the DNA polymerases for the homologous TPs. Among the heterocomplexes, in agreement with the glycerol gradient centrifugation results, the affinity of the \( \phi 29 \) polymerase for NfTP (Fig. 2, lane e) is higher than that of the Nf polymerase for the \( \phi 29 \) TP (Fig. 2, lane i). The assay was performed also with 10 mM Mg\(^{2+}\) as catalytic metal, obtaining the same results; there-
fore, the nature of the cation does not seem to affect the DNA polymerase-TP interaction.

The functionality of these complexes was tested by their ability to deoxynucleotidylate TP in the absence of template (23). Fig. 3 shows that, as expected, homocomplexes are more active than heterocomplexes, being the activity of the Nf homocomplex 64% that of the φ29 counterpart. The activity of the heterocomplexes drops below 10% for the φ29 pol/Nf TP and is negligible for Nf pol/φ29TP. The instability of the φ29 TP/Nf DNA polymerase complex accounts for this low activity, as was shown by glycerol gradient centrifugation (Fig. 1) and competition experiments (Fig. 2, lane i). Therefore, the differences observed in the activity of the heterocomplexes could, at least partially, reflect differences in the stability of the complexes.

**Efficient Initiation of Replication Requires a Specific Recognition**

between DNA Polymerase and TP-DNA—The specificity of the protein-protein interactions involved in recognition of the origins of replication was studied by initiation of replication assays using φ29 and Nf pol/TP homo- and heterocomplexes on both φ29 and Nf TP-DNAs. When homocomplexes of one phage were assayed with TP-DNA of the other, the initiation activity was hardly detected when Mg$^{2+}$ was used as catalytic metal (results not shown). This problem was overcome by using Mn$^{2+}$ instead Mg$^{2+}$, resulting in an increase of activity over 100-fold (not shown). The activity of either φ29 or Nf DNA polymerase/TP homocomplexes on their respective TP-DNAs (Fig. 4, A and B) is higher than that on TP-DNAs from the other phage. Thus, when φ29 TP-DNA was assayed with the φ29 pol/TP homocomplex, the activity detected after a 60-min reaction was 10-fold higher than that of Nf pol/TP homocomplex; moreover, the reaction with the latter was much slower, since this difference was about 200-fold after 5 min (Fig. 4A). On the other hand, when Nf TP-DNA was assayed with the Nf pol/TP homocomplex the activity was also higher than that with the φ29 pol/TP homocomplex (Fig. 4B), but this difference was not as great as the one observed with φ29 TP-DNA; this different behavior could be due to a lower specificity of φ29 initiation proteins for the Nf TP-DNA or to a reduced stringency of the latter. Altogether, these results confirm the specificity between the pol/TP complex and the TP-DNA template.

To determine whether the recognition specificity of origins is provided by the DNA polymerase, the TP, or both, we assayed pol/TP heterocomplexes with φ29 and Nf TP-DNAs as templates. Interestingly, we observed that when the DNA polymerase was from the same phage as the template, namely φ29 pol/Nf TP with φ29 TP-DNA (Fig. 4C) or Nf pol/φ29 TP heterocomplexes (C and D) were used, TP-dAMP formation was represented as arbitrary units derived from the absorbance of scanned bands in exposed gels and therefore comparable among the four panels.

**Initiation of TP-free Origins of Replication**—The contribution of the parental TP in the initiation of protein-primed replication activity was assessed by comparison of φ29 and Nf
TP-DNAs with the corresponding TP-free left origins of replication (oriLs). Fig. 5A shows that the activity of φ29 oriL is 12–20% of the activity of the TP-DNA preparation, as it has been described previously. The same situation was observed for Nf (Fig. 5B), although Nf showed an even higher dependence of parental TP, as Nf oriL activity is about 2–5% that of Nf TP-DNA. Thus, parental TP strongly enhances initiation of replication to a degree that depends on the system.

It had been described that φ29 DNA polymerase and TP initiate replication with Nf oriL as template at about the same rate as with φ29 oriL. Therefore, the specificity of recognition seemed to be lost with TP-free templates. The availability of purified DNA polymerase and TP from Nf, together with the use of Mn²⁺ instead of Mg²⁺ in the in vitro initiation reaction, allowed us to extend these studies measuring the rate of initiation of replication with pol-TP homo- and heterocomplexes with oriLs of φ29 and Nf. With either φ29 (Fig. 6A) or Nf (Fig. 6B) oriLs, the behavior of φ29 and Nf homocomplexes was similar, and the specificity observed with TP-DNAs (see Fig. 5) is lost.

When both templates the reaction with φ29 pol-TP is faster than with Nf pol-TP, reaching a similar level of initiation at later times.

DISCUSSION

Linear DNA genomes with TPs, like that of B. subtilis phage φ29, are replicated by a protein-priming mechanism. The origins of replication, located at the genome ends, are recognized by a complex formed by the DNA polymerase and a free TP that is the primer for the following replication round. Thus, the formation of the initiation complex involves the DNA polymerase and two TPs, one of them free, acting as a primer (primer TP) and the other covalently linked to the DNA 5’ ends (parental TP).

We have used both the proteins and the template DNA from φ29 and the related phage Nf to study the specificity of the initiation reaction.

We first studied the interaction of DNA polymerases with TPs, since the formation of these complexes precedes the initiation of DNA replication. Heterologous DNA polymerases and TPs are able to form complexes, although their interaction, especially that of Nf DNA polymerase and φ29 TP, is weaker than that of the homologous proteins. Since TP binds to the same active site in DNA polymerase as a primer DNA (10), they cannot bind simultaneously, we challenged TP with DNA. The DNA was able to displace heterologous but not homologous TPs from DNA polymerases, indicating that homocomplexes are more stable than heterocomplexes, in agreement with the previous results. Accordingly, the ability of DNA polymerases to deoxyadenylate a heterologous TP in the absence of any template was much lower than that with a homologous TP.

However, their activities may not only reflect the stability of the different pol-TP complexes but also their catalytic efficiency.

φ29 DNA polymerase belongs to the eukaryotic polymerase α family (40, 41) of which the crystal structure of that of bacteriophage RB69, a member of the family, is known (42). The Ser^{222} residue of φ29 DNA polymerase, which has been proposed to be required for TP binding (39), is located in the exonuclease domain, facing the editing channel in the RB69 corresponding region (42). In addition, the motif Y^{226}XG(G/A), which is also involved in TP binding, would be located near the polymerase-active site (43). On the other hand, the R^{256}GD residues of φ29 TP are involved in the interaction with DNA polymerase (44). The regions containing these interacting residues in both DNA polymerase and TP are highly conserved in Nf. Nevertheless, these phases may have evolved to get slight structural differences in their DNA polymerases and TPs that justify the preferential binding to their own partner.

Once the pol-TP complex is formed, it must specifically recognize the origins of replication. It has long been acknowledged as the critical role of the parental TP in the initiation reaction.

The lack of activity of φ29 pol-TP with TP-DNA templates from a different group of related phages, such as Nf (22), was indicative of the high specificity of the reaction. The use of Mn²⁺, about a 100-fold more efficient activator of initiation
than Mg$^{2+}$ (Ref. 29 and this work), allowed detection of initiation using φ29 pol·TP complexes with Nf templates. Initiation activity with both φ29 and Nf TP-DNAs was measured using φ29 and Nf initiation proteins. As expected, pol·TP homocomplexes were more active with their own template. When pol·TP heterocomplexes were used, we found that the only type of template significantly active for initiation of replication was the TP-DNA from the same phage as the DNA polymerase. Since parental TPs readily interact with each other (45), it was assumed that primer TP would direct the pol·TP complex to the parental TP (44, 46). Instead, our results point out that the specificity to the template. In addition, these results further support the TP-DNA from the same phage as the DNA polymerase.

The recognition of parental TP by the DNA polymerase complexed with primer TP is essential for an efficient initiation of DNA replication. However, these results do not rule out a recognition between parental and primer TP, as has been recently proposed (47).

The selective recruitment of pol·TP complexes at the TP-containing replication origins could provide the correct positioning of primer TP at the active site of polymerase. Nevertheless an optimized rate of initiation requires highly specific protein interactions and is only obtained when DNA polymerase is homologous to TP-DNA and primer TP. In fact, in vivo studies have shown a strict requirement for a primer TP homologous to TP-DNA and/or DNA polymerase (28, 48).

Parental TP is essential for the efficient initiation of replication, as TP-free templates are much less active than TP-DNAs. Furthermore, TP-free templates show a similar activity with φ29 and Nf pol·TPs, indicating that parental TP provides specificity to the template. In addition, these results further support the hypothesis that the nucleotide sequence by itself does not play a major role in the specific recognition of origins by the initiation proteins.

In conclusion, we can envisage a scenario for initiation of protein-priming replication in which the specificity of recognition of the replication origins is determined by the parental TP. The DNA polymerase engaged in a complex with primer TP specifically recognizes the parental TP. This recognition would result in a higher affinity binding of the pol·TP complexes to the origins and/or in an increase of their catalytic efficiency of the initiation reaction.

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