Phage φ29 Terminal Protein Residues Asn\textsuperscript{80} and Tyr\textsuperscript{82} Are Recognition Elements of the Replication Origins

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Initiation of phage φ29 DNA replication starts with the recognition of the origin of replication, located at both ends of the linear DNA, by a heterodimer formed by the φ29 terminal protein (TP) and the φ29 DNA polymerase. The parental TP, covalently linked to the DNA ends, is one of the main components of the replication origin. Here we provide evidence that recognition of the origin is mediated through interactions between the TP of the TP/DNA polymerase heterodimer, called primer TP, and the parental TP. Based on amino acid sequence comparisons, various φ29 TP mutants were generated at conserved amino acid residues from positions 61 to 87.

In vitro φ29 DNA amplification analysis revealed that residues Asn\textsuperscript{80} and Tyr\textsuperscript{82} are essential for functional interaction between primer and parental TP required for recognition of the origin of replication. Although these mutant TPs can form functional heterodimers with φ29 DNA polymerase that are able to recognize the origin of replication, these heterodimers are not able to recognize an origin containing a mutant TP.

DNA replication is a semiconservative process in which a DNA polymerase uses one DNA strand as a template for the synthesis of its complementary strand. All DNA polymerases require a preexisting primer to initiate DNA synthesis (1). In many cases, the primer is a short RNA or DNA molecule. In linear DNAs, which are unable to form circular or hairpin structures during replication, replication of the genome ends cannot take place via RNA or DNA priming. Several mechanisms have evolved to solve the problem of initiation of DNA replication in such linear genomes (2). In the case of viral linear genomes, the OH group of a serine, threonine, or tyrosine residue of a terminal protein (TP)\textsuperscript{1} molecule serves as a primer for DNA replication, and as a consequence, the TP molecule becomes covalently attached to the 5′-terminal nucleotide. This mechanism of initiation of replication is called protein priming (reviewed in Ref. 3). Once bound to the DNA, the TP may serve additional functions such as assisting in DNA packaging (4, 5), protection against nucleases (6), enhancement of infectivity (7, 8), stimulation of template activity (9), matrix attachment (10), stimulation of transcription (10), and recruitment of a new TP to the origin (11). As a step previous to the initiation of replication, TP interacts with the DNA polymerase to form a heterodimer (12). To distinguish between the different functions of TP, we will refer to the TP bound to the DNA ends as parental TP and the TP in the heterodimer with the DNA polymerase as primer TP. Interaction of primer TP and/or DNA polymerase with parental TP may increase the affinity of the TP/DNA polymerase heterodimer for the origin or could assist the latter for its correct positioning at the origin, which may be important for initiation of DNA replication at the correct position.

The protein-priming mechanism of DNA replication has been mainly studied in the Bacillus subtilis bacteriophage φ29 and adenoviruses (see Refs. 2, 3, 13, and 14 for review). The φ29 genome is a 19,285-base pair linear double-stranded DNA molecule (15) with a phage-encoded 31-kDa TP covalently attached to the 5′ termini (16). Genetic studies and the development of an in vitro DNA replication system (17) led to the identification of the origins of replication at each end of the DNA molecule (18, 19). To activate the initiation of replication, dsDNA-binding protein p6 forms a nucleoprotein complex that would help to open the DNA ends (20). A primer TP/DNA polymerase heterodimer recognizes the origin of replication, probably through protein-protein interaction of the primer TP and/or φ29 DNA polymerase with the parental TP. Then, the φ29 DNA polymerase catalyzes the addition of the first dAMP (21) to the OH group provided by Ser\textsuperscript{232} of the primer TP (22). The formation of this first TP-dAMP is directed by the second nucleotide at the 3′-end of the template and then slides back one nucleotide to recover the terminal nucleotide (23). Following initiation, the same φ29 DNA polymerase molecule completes replication of the parental strand.

φ29 parental TP is an important requirement for in vitro initiation of φ29 DNA replication (24). Two lines of evidence show that parental TP is also required in vivo. First, in a mixed infection experiment at 42 °C using phages that have a thermosensitive (ts) mutation in either gene 2 (encoding the DNA polymerase) or gene 3 (encoding TP), most of the phage progeny had the ts2 genotype (16), and second, successful transfection required an intact gene 3 product (17, 25). Moreover, formation of the TP-dAMP initiation complex was obtained using as template the TP-DNA isolated from the closely related Bacillus phage ø15 but not from the more distantly related Bacillus phage GA-1 or the pneumococcal phage Cp-1. The lack of activity in the initiation reaction of the TP-DNA isolated from these latter two phages could be because of differences in the parental TPs (24).

Structure-function studies and biochemical characterization of φ29 TP provide a basis to gain insight about the different roles of this protein in φ29 DNA replication. As shown here, a
and mutant alkylammonium chloride, which was purchased from Bayer. Wild-type materials for electron microscopy were from Balzers except benzyldimethylprimers were from Roche Molecular Biochemicals. Reagents and materials: TP-DNA was obtained as described (17). Proteinase K and DNA unlabeled nucleotides were obtained from Amersham Pharmacia Bio-theses.

Multiple alignment of the TP amino acid sequences of Bacillus sp. and E. coli bacteriophages. EMBL data base accession number are given in parentheses: ø29 (J02479), PZA (M11813), NF (Y00363), B103 (X99260), GA1 (X96867), and the E. coli bacteriophage PRD1 (M22161). Numbers at the beginning of the amino acid sequence refer to the position in the protein sequence. Black boxes enclose residues conserved in all sequences compared, and gray boxes enclose residues that are only conserved in the TPs of Bacillus bacteriophages. The following amino acids are considered conservative: S and T; A and G; K and R; D, E; Q, and N; I, L, M, V, Y, and F. The residues of ø29 TP that have been subjected to mutagenesis are indicated with an asterisk.

| Table 1 | Functions of wild-type and mutant ø29 TPs |
|---|---|
| ø29 TP | TP-dAMP formation 1 | TP-DNA replication 1 | TP-DNA amplification 1 |
| wt | 100 | 100 | 100 | 23.5 |
| K61M | 92 | 107 | 86 | 26.3 |
| K61R | 137 | 84 | 85 | 25.2 |
| K61T | 70 | 77 | 77 | 28.2 |
| N80S | 85 | 110 | 84 | 2.7 |
| Y82L | 92 | 120 | 76 | 2.3 |
| Y82S | 65 | 90 | 74 | 2.2 |
| G83D | 93 | 108 | 89 | 26.5 |
| S87R | 82 | 60 | 94 | 24.7 |

* Numbers indicate the activity of mutant TPs with respect to the wild-type TP.

** orf29 is a single-stranded 29-mer oligonucleotide with the sequence corresponding to the right ø29 DNA end.

Site-directed Mutagenesis and Expression of ø29 TP Mutants—The wild-type ø29 TP gene, cloned into M13mp18, was amplified using the primer 5′-GAC ATC GAA TTC TAT TCA GAA GGT G-3′ (sense) and 5′-CAT ATG CTG GAT CCT TTA ACG GAG C-3′ (antisense). The site-directed mutagenesis was carried out by polymerase chain reaction using the primer 5′-GAC GCT GTC ATC CAC CAA TTG C-3′ for mutant K61M, 5′-GAC GCT GTC TCC TTC CAG CCA TTA TTG-3′ for mutant K61R, 5′-GAC GCT GTC TCC TTC CAG CCA TTA TTG-3′ for K61T, 5′-CAC ACC GTG CTT TTC GAA C-3′ for N80S, 5′-CAC ACC TAA TCG ATT CTT TTC G-3′ for Y82L, 5′-CAC ACC AGG GGA TGC ATT CTT TTC G-3′ for Y82S, 5′-CTA GCC ACC ACA TCG TAT GCA TTC-3′ for G83D, and 5′-CTT GTC TTT ACT AGC CAC CAC ACC GAC-3′ for S87R. The polymerase chain reaction fragments carrying the different mutations were subcloned in plasmid pA23e3a (28), which expresses ø29 TP under the control of the λ P1 promoter. The presence of the desired mutations and the absence of additional mutations were confirmed by sequencing each ø29 TP mutant gene using the primers: 5′-GAC ATC GAA TTC TAT TCA GAA GGT G-3′ (sense) and 5′-CAT ATG CTG GAT CCT TTA ACG GAG C-3′ (antisense). Sequencing was carried out by the chain termination method (29), using Sequase kit version 2.0 from U. S. Biochemical Corp. Expression of the mutant proteins was carried out in the Escherichia coli strain N1P1. ø29 TP-dAMP Formation (Protein-primed Initiation Assay)—The incubation mixtures contained (in 25 μl) 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 20 mM ammonium sulfate, 4% glycerol, 0.1 mg/ml bovine serum albumin, 0.25 mM [α-32P]dATP (2.5 μCi), 0.5 μg of ø29 TP-DNA, or 0.2 μg of single-stranded 29-mer oligonucleotide with the sequence corresponding to the right ø29 DNA end (orf29), 5′-AAA GTA GGG TAC ACC GAC AAC ATA CAC CA-3′, 10 ng of wild-type or

Fig. 1. Multiple alignment of the TP amino acid sequences of Bacillus sp. and E. coli bacteriophages. EMBL data base accession number are given in parentheses: ø29 (J02479), PZA (M11813), NF (Y00363), B103 (X99260), GA1 (X96867), and the E. coli bacteriophage PRD1 (M22161). Numbers at the beginning of the amino acid sequence refer to the position in the protein sequence. Black boxes enclose residues conserved in all sequences compared, and gray boxes enclose residues that are only conserved in the TPs of Bacillus bacteriophages. The following amino acids are considered conservative: S and T; A and G; K and R; D, E; Q, and N; I, L, M, V, Y, and F. The residues of ø29 TP that have been subjected to mutagenesis are indicated with an asterisk.

Fig. 2. In vitro ø29 TP-DNA replication. The assays were carried out in the presence of 10 ng of either ø29 wild-type (wt) or mutant TPs and 20 ng of ø29 DNA polymerase (see “Materials and Methods”). After incubation for the indicated times at 30 °C, the samples were first used to calculate their relative replication activity by measuring the amount of incorporated [α-32P]dAMP (see Table I). Next, the samples were run in an alkaline agarose gel to determine the lengths of the synthesized DNAs. The migration position of unit length ø29 DNA is indicated by bp, base pairs.
As described under "Materials and Methods," in the presence of 20 ng of φ29 DNA polymerase and 10 ng of the indicated TP, the amount and size of amplified DNA was analyzed by alkaline agarose gel electrophoresis followed by ethidium bromide staining. The amount of input DNA is shown as control (C). wt, wild-type.

RESULTS AND DISCUSSION

Single Substitutions at Residues Lys61, Asn80, Tyr82, Gly83, and Ser87 in φ29 TP Do Not Affect Its Function as a Primer—A multiple alignment of the amino acid sequence of TPs of Bacillus sp. phages φ29, PZA, Nf, B103 and GA-1, and TP of the E. coli phage PRD1 is presented in Fig. 1. These TPs are very similar in size, ranging from 258 (phage PRD1) to 267 (phage Nf) amino acids. The percentage of identical amino acids shared by these TPs is 9% (22% similarity) increasing to 32% (52% similarity) when only the TPs of Bacillus sp. are considered. Nonetheless, the TPs from the Bacillus sp. phages and from the E. coli phage PRD1 share a significant number of conserved amino acids, some of which may be important for its protein structure and/or function.

To study the function of the most conserved residues in the TP region from position 61 to 87, single changes were introduced in two φ29 TP residues that are invariant in all the aligned TPs shown in Fig. 1 (Lys61 and Ser87) and in three additional residues that are invariant in the TPs of the Bacillus phages (Asn80, Tyr82, and Gly83). The changes were designed taking into account secondary structure predictions (32, 33) and general suggestions for conservative substitutions (34).

Eight mutants were obtained K61M, K61R, K61T, N80S, Y82L, Y82S, G83D, and S87R. Site-directed mutagenesis, overproduction, and purification of the mutant proteins were carried out as described under "Materials and Methods."

Replication of φ29 TP-DNA starts at both ends by a specific protein-priming mechanism. In this process the viral DNA polymerase, which forms a heterodimer with the φ29 TP, catalyzes the linkage of dAMP to the TP, which acts as a primer. To evaluate the primer function of mutant TPs, we studied the formation of the TP-dAMP initiation complex (initiation reaction) using as a template a single-stranded 29-mer oligonucleotide with the sequence corresponding to the right φ29 DNA end (oriR(29)) but lacking parental TP (see "Materials and Methods"). As shown in Table I, the amount of dAMP incorporated to the various mutant TPs was comparable to that of the wild-type TP, indicating that these mutant TPs interact with φ29 DNA polymerase and that they are able to serve as a primer in a reaction that does not involve interactions with parental TP.

To determine whether the presence of parental TP affects the primer function of the mutant TPs, the in vitro formation of the TP-dAMP initiation complex using φ29 TP-DNA as a template was assayed with wild-type or the mutant TPs. The results were similar to those obtained when an oligonucleotide was used as a template (see Table I). Therefore, the interactions between the mutant TP/DNA polymerase heterodimer and the wild-type parental TP, which probably take place during the
recognition of the origin in the initiation of replication, are not affected by these mutations.

After the formation of the TP-dAMP initiation complex, the DNA polymerase remains associated with the primer TP until a short DNA primer of 9 nucleotides has been formed. It is only after this so-called transition step that the two proteins dissociate, and the DNA polymerase continues elongation until complete replication of the nascent DNA chain is achieved (35). To study possible effects of the mutations introduced in the TP on the transition step, we carried out replication assays in which one full round of replication is allowed (see “Materials and Methods”). As shown in Fig. 2, the amount of replicated DNA and the velocity of the reactions were similar when either wild-type or mutant TPs was used (see also Table I). Therefore, the single amino acid changes introduced in the TP do not affect the dissociation of the TP/DNA polymerase heterodimer needed to proceed into elongation in the DNA replication process.

These results suggest that the mutant TPs are able to serve as a primer with an efficiency comparable to that of wild-type TP indicating that the residues Lys61, Asn80, Tyr82, Gly83, and Ser87 are not involved in the primer activity of the TP, and they are not involved in the transition step.

Mutations at the Asn80 and Tyr82 Residues Alter the Parental TP Function—An in vitro φ29 DNA amplification system has been described (36) that requires the following purified proteins of φ29: TP, DNA polymerase, the dsDNA-binding protein p6, and the ssDNA-binding protein p5. During the first round of φ29 TP-DNA amplification the TP present in the TP/DNA polymerase heterodimer acts as a primer and becomes covalently linked to the newly synthesized DNA strand. In subsequent replication rounds this TP molecule will act as a parental TP. This in vitro assay mimics the natural amplification of viral DNA. To analyze the possible effects of the mutations introduced in the TP on its function as parental TP, DNA amplification assays were carried out as described under “Materials and Methods.” Fig. 3 shows the amount of DNA synthesized using wild-type or mutant TPs. Mutants K61M, K61R, K61T, G83D, and S87R led to levels of amplification similar or even higher than wild-type TP (see also Table I). However, mutants N80S, Y82L, and Y82S were very inefficient in the amplification reactions despite the fact that they have wild-type-like primer activity. To determine in which stage of the amplification process these mutant TPs are affected, the syn-
thesis of DNA was studied as a function of time. DNA amplification experiments were carried out as described above with wild-type and mutant TPs N80S, Y82L, or Y82S. As shown in Fig. 4, the amount of DNA synthesized increased during the first 10 min, using as primer either the wild-type or mutant TPs. Afterward, DNA synthesis primed by the wild-type TP continued increasing with time, whereas DNA synthesis primed by mutant TPs begins to reach a plateau after 20 min. Analysis by native agarose gel electrophoresis showed that, in all cases, unit length \( {\phi}29 \) DNA was synthesized (Fig. 4B). The amount of \( {\phi}29 \) synthesized DNA (in nanograms) was quantified from the amount of radioactivity incorporated into DNA (Fig. 4A), and the amplification factor was calculated as the ratio between the amount of DNA at the end of the reaction (input DNA plus synthesized DNA) and the amount of input DNA. The data, shown in Table II, indicate that the amount of \( {\phi}29 \) synthesized DNA initiated with these mutant TPs implies an amplification factor close to 3 and never exceeded this value even after extended incubation times. Assuming that all DNA molecules were initiated and replicated completely, a 2-fold amplification factor is expected after the first round of replication in a normal process, becoming a 4-fold amplification factor after completion of the second round (see Fig. 5A). Control experiments with the wild-type TP showed amplification levels close to 30-fold (Table II). However, with mutant TPs N80S, Y82L, or Y82S the amplification factor only reached a maximum of 3, indicating that DNA synthesis was stalled in some way before completing a second round of replication. These results are explained in the scheme shown in Fig. 5B. After a wild-type TP-DNA molecule has been replicated in an \( {\text{in vitro}} \) experiment containing a mutant TP in the reaction mixture, both daughter molecules generated contain one origin of replication with a wild-type TP and the other with a mutant TP. If the presence of the mutant TP inactivates the corresponding origin, the second round of replication will be 50% productive because only the origin with a wild-type TP could be used for initiation. Therefore, if this model is correct, 3-fold is the maximal amplification factor expected in the assay. This is in agreement with the data obtained in the quantitation of the DNA synthesized \( {\text{in vitro}} \) (see Table II). Moreover, replication would stop after this incomplete second replication round because now all double-stranded origins would contain a mutant TP, being the DNA strands corresponding to the input DNA fully displaced. The possibility that the two displaced parental strands could hybridize to reconstitute a molecule with two active origins is precluded by the presence of \( {\phi}29 \) ssDNA-binding protein in the assay.

Detection of Single-stranded DNA Molecules as a Consequence of the Inactivation of the Replication Origins—The explanation depicted in Fig. 5B predicts the generation of full-size displaced ssDNA. To detect the presence of these ssDNA molecules, samples of the amplification assays (after 40 min) were subjected to electron microscopy analysis (see “Materials and Methods”). To distinguish between dsDNA and ssDNA molecules, a psoralen cross-linking procedure was used (30). It has been shown that psoralen treatment of \( {\phi}29 \) DNA in the presence of protein p6 followed by short UV light irradiation and DNA spreading under denaturing conditions produces molecules that contain small bubbles corresponding to ssDNA regions, in which the binding of p6 prevents psoralen cross-linking (37). Taking into account that protein p6 binds to dsDNA and not to ssDNA and that \( {\phi}29 \) ssDNA-binding protein covers the displaced strand of the replicative intermediate, molecules without bubbles correspond to displaced ssDNA. Fig. 6A shows a typical example of a replicative intermediate taken from a DNA amplification assay using the wild-type TP, and it consists of a dsDNA with one ssDNA branch that corresponds to the displaced strand from one origin. In addition to molecules with a single ssDNA branch, DNA molecules were observed with two ssDNA branches. However, no full-length \( {\phi}29 \) ssDNA molecules were observed in these samples (results not shown). Electron microscopy analysis of DNA amplification products using mutant TPs N80S, Y82L, or Y82S revealed the appearance of full-length \( {\phi}29 \) ssDNA molecules as shown in Fig. 6B for the mutant TP Y82L.

Previous analysis of the different types of replicative intermediate molecules synthesized during \( {\phi}29 \) DNA replication indicated that neither \( {\text{in vivo}} \) (18) nor \( {\text{in vitro}} \) (38) full-length \( {\phi}29 \) ssDNA molecules were detected when natural \( {\phi}29 \) DNA template with TP at both DNA ends was used. Full-length ssDNA molecules were only observed in \( {\text{in vitro}} \) replication assays using recombinant \( {\phi}29 \) templates lacking TP at one end (38). Full-length \( {\phi}29 \) ssDNA molecules may also be generated when the initiation rate is reduced leading to only one initiation event/dsDNA molecule. However, as shown in Fig. 4, ini-
tiation rates of mutant TPs are similar to that of wild-type TP (at short reaction times). Therefore, the finding of full-length ssDNA molecules favors the model presented above in which an origin containing one of the mutant TPs N80S, Y82L, or Y82S is inactive.

**Addition of Wild-type TP/DNA Polymerase Heterodimer Counteracts the Inactive Origin**—The above results show that mutations at residues N80 or Y82 of φ29 TP do not affect the function of primer protein but alter function as parental TP. Thus, a mutant TP/DNA polymerase heterodimer is able to recognize a wild-type origin allowing a first round of DNA replication. However, once the TP becomes parental TP the origin is inactive. This inactivation could be because of: (i) the involvement of these parental TP residues in a “one side” interaction with the primer TP/DNA polymerase heterodimer or (ii) a reciprocal (“two side”) involvement of these residues of the parental-primer TP interactions. To distinguish between these two possibilities, we carried out the following experiments. After amplification for 40 min, when mutant primer TPs became mutant parental TPs and DNA synthesis stops, new TP/DNA polymerase heterodimers were added to the amplification reaction (Fig. 7). Upon the addition of wild-type TP/DNA polymerase heterodimer, renewed incorporation of dAMP was observed. On the contrary, addition of heterodimers with mutant TPs did not allow dAMP incorporation. These results indicate that the origin containing a mutant parental TP can be recognized (rescued) by a wild-type TP/DNA polymerase heterodimer but not when it contains a mutant TP. Therefore, the mutations introduced are critical when they are simultaneously present in both the primer and the parental TP, probably affecting their proper interaction required for a functional initiation reaction. To test this possibility, an amplification assay was carried out for 40 min using mutant TP N80S to obtain DNA molecules containing mutant TP at their origins. These products were then used as templates in initiation reactions with either wild-type or mutant TP/DNA polymerase heterodimers. Initiation reactions were only obtained with the wild-type heterodimer (results not shown). The capacity of the wild-type TP/DNA polymerase to rescue a mutant TP/DNA origin, both in amplification and initiation assays, seems to rule out the possibility that a mutant TP/DNA polymerase heterodimer forms an abortive (nonproductive) complex that blocks the mutant TP/DNA origin. Therefore, we consider that the inactivation of the origin is caused by the lack of functional interactions between mutant primer and mutant parental TP.

**Conclusion**—Single substitutions at conserved residues Lys$^{81}$, Asn$^{80}$, Tyr$^{82}$, Gly$^{83}$, and Ser$^{87}$ of φ29 TP did not alter its primer function either in the presence or in the absence of parental TP in the template, indicating that these residues are not involved in (i) the interaction with φ29 DNA polymerase and (ii) the formation of the linkage to dAMP. Furthermore, these mutant TPs were not affected in the transition step, a necessary step leading to TP/DNA polymerase dissociation to enable processive DNA polymerization. However, once the mutant primer TP became parental TP the replication origin was inactivated to initiate additional replication rounds. Altogether, the results presented in this paper indicate that recognition of φ29 DNA replication origins occurs through direct interactions between primer and parental TP. In addition, we have provided evidence that residues Asn$^{80}$ and Tyr$^{82}$ of φ29 TP are essential for such functional interactions at the origins. Moreover, for the first time we have found mutations that do not affect the individual function of the TP either as primer or as parental but are critical when present simultaneously in the primer and parental TPs. These results support the existence of recognition elements at the φ29 DNA replication origin that imply complementary interactions between primer and parental TP.

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