The linear double-stranded genome of phage \( \phi29 \) contains a terminal protein (TP) covalently linked at each 5' DNA end, called parental TP. Initiation of \( \phi29 \) DNA replication starts with the recognition of the origins of replication, constituted by the parental TP-containing DNA ends, by a heterodimer containing \( \phi29 \) DNA polymerase and primer TP. It has been argued that origin recognition involves protein-protein interactions between parental and primer TP. Analysis of the TP sequence revealed that the region between amino acids 84 and 118 has a high probability to form an amphipatic \( \alpha \)-helix that could be involved in the interaction between parental and primer TP. Therefore, this TP region may be important for origin recognition. To test this hypothesis we introduced various mutations in the predicted amphipatic \( \alpha \)-helix and analyzed the functionality of the corresponding purified TP mutants. The results obtained show that the identified putative amphipatic \( \alpha \)-helix of TP is an important determinant involved in origin recognition.

Before DNA polymerase duplicates a DNA strand using its complementary strand as template, DNA replication has to be initiated. This process involves several distinct steps such as recognition of the origins, unwinding of double-strand (ds) DNA, and priming (for reviews, see Refs. 1 and 2). Genomes consisting of a linear dsDNA molecule with a terminal protein (TP) covalently linked to their 5'-ends have been found in bacteriophages (e.g. \( \phi29 \), \( \phi15 \), Nf, B103, GA-1, Cp-1, and PRD1), animal viruses (e.g. adenoviruses), plasmids (e.g. S1 and Kalli), and bacteria (e.g. Streptomyces). In most of these cases, initiation of replication has been shown to occur via a so-called protein-priming mechanism, which has been studied extensively for the Bacillus subtilis phage \( \phi29 \) (for reviews, see Refs. 3 and 4). Fig. 1 shows a schematic representation of in vitro \( \phi29 \) DNA replication. Initiation of \( \phi29 \) DNA replication starts with recognition of the origin of replication, i.e. the TP-containing DNA ends, by a TP/DNA polymerase heterodimer. The TP linked to the 5' DNA ends is called parental TP, and the TP present in the complex with DNA polymerase is called primer TP. The viral ds DNA-binding protein p6 forms a nucleoprotein complex that would help to open the DNA ends (5), facilitating the formation of the covalent linkage between dAMP and the OH group of Ser\(^{225} \) residue of the primer TP, catalyzed by \( \phi29 \) DNA polymerase (6, 7). The formation of this first TP-dAMP covalent complex is directed by the second nucleotide at the 3'-end of the template; then, the TP-dAMP complex slides back one nucleotide to recover the information of the terminal nucleotide (8). Next, the \( \phi29 \) DNA polymerase synthesizes a short elongation product before dissociating from the TP (9). Replication, which starts at both DNA ends, is coupled to strand displacement. This results in the generation of so-called type I replication intermediates consisting of full-length double-stranded \( \phi29 \) DNA molecules with one or more single-stranded DNA branches of varying lengths. When the two converging DNA polymerases merge, a type I replication intermediate becomes physically separated into two type II replication intermediates. Each of these consists of a full-length \( \phi29 \) DNA molecule in which a portion of the DNA, starting from one end, is double-stranded and the portion spanning to the other end is single-stranded (10, 11). Continuous elongation by the DNA polymerase completes replication of the parental strand.

The \( \phi29 \) DNA polymerase and TP form a stable heterodimer (12). This complex recognizes the origin of replication, probably through protein-protein interactions between primer and parental TP (13, 14). Secondary structure predictions of \( \phi29 \) TP showed that the region between residues 84 and 118 has a high probability to form a coiled coil structure. A coiled coil is a structural motif formed between two or more amphipatic \( \alpha \)-helices (15–17). The coiled coil motif is characterized by a heptad repeat denoted \( abcddefg \). Residues \( a \) and \( d \) are normally occupied by hydrophobic residues (18) falling on the same side of the helix and forming the hydrophobic interface between two (or more) helices. These hydrophobic interactions provide the major driving force for formation and stability of the coiled coil (19–21). The residues located at the opposite face participate in intrahelical interactions. These positions generally contain charged residues, which may lead to intrahelical electrostatic attractions or repulsions, thereby stabilizing or destabilizing the coiled coil (22). Thus, coiled coil motifs are often important structures for the formation of protein di- or multimerization. The identified putative coiled coil region in the \( \phi29 \) TP may provide an appropriate surface for the interaction between parental and primer TP and, hence, it may have an important role in the recognition of the origin of replication.
test this hypothesis we constructed site-directed mutants of \( \phi 29 \) TP in which hydrophobic residues located at position \( a \) of the predicted amphipatic \( \alpha \)-helix were substituted by alanine. In addition, mutants were constructed in which positively charged residues located at position \( f \) of the predicted amphipatic \( \alpha \)-helix were substituted by the negatively charged residue glutamic acid. The functionality of the purified mutant TPs was analyzed by different in vitro \( \phi 29 \) DNA replication assays; i.e., initiation, elongation, and amplification. The results obtained indicate that the region of \( \phi 29 \) TP predicted to form an amphipatic \( \alpha \)-helix is important for recognition of the origin of replication.

MATERIALS AND METHODS

Bacterial Strains—Escherichia coli strain JM109 (F' ralD36 lacIq (lacZM15 proA+ B' +/e14-McrA-)/lac-proAB thi gyrA96 (Nalr) endA1 hsdR17 (rK-mk- +) relA1 supE44 recA1; Ref. 23) was used for cloning and overexpression of wild-type and mutant TPs. Epicurian coli™ XL1-blue supercompetent cells were used in combination with the QuickChange™ site-directed mutagenesis kit. When necessary, chloramphenicol and ampicillin were added to E. coli cultures at final concentrations of 10 and 100 \( \mu \)g/ml, respectively.

DNA Techniques—All DNA manipulations were carried out according to Sambrook et al. (24). Restriction enzymes were used as indicated by the suppliers. [\( \alpha ^{32}P \)]dATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. TP-DNA was prepared as described (25). DNA fragments were isolated from agarose gels using the Qiaex Gel Extraction Kit (Qiagen Inc., Chatsworth, CA). The dideoxynucleotide DNA fragments were isolated from agarose gels using the Qiaex Gel Extraction Kit (Qiagen Inc., Chatsworth, CA). The dideoxynucleotide DNA fragments were isolated from agarose gels using the Qiaex Gel Extraction Kit (Qiagen Inc., Chatsworth, CA). The dideoxynucleotide DNA fragments were isolated from agarose gels using the Qiaex Gel Extraction Kit (Qiagen Inc., Chatsworth, CA).

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Polymerase Chain Reaction Techniques—The QuickChange™ site-directed mutagenesis kit (Stratagene) was used as indicated by the supplier for the introduction of mutations in \( \phi 29 \) gene 3, encoding TP. Other polymerase chain reactions were carried out essentially as described (27) using the proofreading-proficient Vent DNA polymerase (New England Biolabs, Beverly, MA). In these cases template DNAs were denatured for 1 min at 94 °C. Next, DNA fragments were amplified in 30 cycles of denaturation (30 s, 94 °C), primer annealing (1 min; 600 °C), and DNA synthesis (3 min, 72 °C). Oligonucleotides (listed in Table I) were purchased from GENSET.

Construction and Purification of His-tag Labeled \( \phi 29 \) TP Mutants—The wild-type \( \phi 29 \) TP gene was amplified using the primers Alex1 and Alex2 (see Table I) and \( \phi 29 \) DNA as template. The polymerase chain reaction product obtained was purified, digested with BamHI and NotI, and cloned into pBluescript II Ks, digested previously with the same enzymes. The resulting plasmid was named pBluescript + p3. Next, the Quickchange™ site-directed mutagenesis kit was exploited to introduce mutations in gene 3 using the appropriate primers (Table I) and pBluescript + p3 as template DNA. The presence of the desired mutations and the absence of additional mutations in the resulting plasmids were confirmed by sequence analysis. Next, each of the BamHI-NotI fragments containing either the wild-type \( \phi 29 \) gene 3 or those with the desired mutations was isolated and cloned into the corresponding restriction sites of pUSH1 (26). As a result, the various gene 3 regions were cloned in-frame behind the His-tag encoding sequence of pUSH1. The absence of additional mutations in the resulting plasmids was confirmed by sequence analysis. The E. coli strain JM109 was used as a host to overexpress the various His-tag labeled mutants of TP. For this purpose, overnight cultures were diluted 100-fold in fresh prewarmed LB-medium and grown to an \( A_{600} \) of 0.6 to 0.7. Expression, induced upon isopropyl-1-thio-\( \beta \)-galactopyranoside addition to a final concentration of 1 mM, was allowed for 2 h. Cells were harvested by centrifugation and stored at \(-70^\circ\)C until further use. Frozen cells were thawed at 4 °C and ground with twice their weight of alumina powder (Merck) for 20 min. The slurry was resuspended in buffer A (50 mM Tris-\( \mathrm{HCl}, \mathrm{pH} \) 7.3, 0.5 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, and 7 mM \( \beta \)-mercaptoethanol) using 4 volumes per gram of cells. To remove the alumina and intact cells the mixture was centrifuged at 2,500 × g. The pellet was resuspended in 2 volumes of buffer A and centrifuged again as before. The pooled supernatants were next centrifuged for 15 min at 15,000 × g to pellet the insoluble proteins. These insoluble proteins were reisolated in 2 volumes of 2.5 g guanidine chloride and centrifuged for 30 min at 30,000 × g. The supernatant was first dialyzed against buffer A containing 1 mM NaCl, and then against buffer B (50 mM NaPO₄, buffer, pH 7.8, 0.5 mM NaCl, 3 mM \( \beta \)-mercaptoethanol). The cell extract was centrifuged again for 15 min at 15,000 × g and the supernatant was subsequently passed twice over a 1-ml Ni²⁺-NTA resin column equilibrated in buffer B. Next, the column was washed with at least 10 column volumes of buffer B containing 20, 30, 40, and 45 mM imidazole, respectively. The recombinant protein was then eluted with

![FIG. 1. Mechanism of in vitro \( \phi 29 \) DNA replication. See text for details. Parental and primer TP are indicated with filled and open circles, respectively. Triangles represent DNA polymerase. Synthesized DNA strands are indicated with broken lines. For clarity only one of the two origins of replication is shown in B.](http://www.jbc.org/)

### Table I

| Name     | Sequence (5’-3’)                     |
|----------|--------------------------------------|
| Alex-1   | caaaggatetcggagaagtcagtagataagc      |
| Alex-2   | caaagggggcggctacccctattaagtttagact  |
| p3-R103E-a,b | egtaacaaggaagttcagggttagttgagag   |
| p3-R110A-a,b | eggttagatagctagaaatctggattagaaagc |
| p3-V84A-a,b | gaatgtagatctggtggctaaaagc           |
| p3-I91A-a,b | ggtggtttagtaaatgtagctagtgattagaaagc|
| p3-V105A-a,b | gttagcggttagcagtgaaatccaggc         |
| p3-M112A-a,b | gtagagaaataatggctggagaaaaaagcagtc   |
| p3-R96E-a,b  | gtagatggtagagcaggaacaaagaggg        |
5 ml of buffer B containing 200 mM imidazole. Finally, the eluate was dialyzed against buffer A containing 200 mM NaCl and 50% glycerol, and the protein was stored at −70 °C in aliquots.

**Glycerol Gradients—** Wild-type or mutant TPs were incubated, in 0.25 ml, with d29 DNA polymerase (5 and 10 μg of each protein, respectively; see Materials and Methods) in a buffer containing 50 mM Tris-HCl, pH 7.5, 20 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, and 20 μg of BSA. The samples were loaded on top of linear 15–30% glycerol gradients (4 ml) containing 50 mM Tris-HCl, pH 7.5, 0.45 mM NaCl, 20 mM ammonium sulfate, 2 mM ZnSO₄, 1 mM EDTA, and 7 mM β-mercaptoethanol. In addition, 20 μg of BSA was loaded on the gradients to serve as internal molecular weight marker. The gradients were centrifuged for 24 h at 58,000 rpm in a TST 60.4 Beckman rotor, at 4 °C. After centrifugation the gradients were fractionated from the bottom and aliquots of each fraction were subjected to SDS-PAGE. TP and DNA polymerase were detected by Western blotting.

**d29 TP-dAMP Formation (Protein-primed Initiation Assay)—** The incubation mixtures contained (in 25 μl) 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 20 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml BSA, 0.1 μM [32P]-dATP (1 μCi), 0.5 μg of d29 TP-DNA, the indicated amount of wild-type or mutant d29 TP, and 10 ng of d29 DNA polymerase. After incubation for 5 min at 30 °C, in conditions shown to be linear with time and enzyme amount, the reactions were stopped by adding EDTA to 10 mM and SDS to 0.1%. The samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The excluded volumes were analyzed by SDS-PAGE and autoradiography. The relative amounts of incorporated [32P]-dAMP were calculated by densitometric analysis of the autoradiographs. In the case of the template-independent assays, TP-DNA was omitted from the reaction mixtures and 1 mM MnCl₂ was used as metal activator instead of MgCl₂.

These latter reactions were carried out for 12 h at 4 °C. 

**d29 TP-Replication Assay (Protein-primed Initiation plus Elongation)—** The incubation mixtures contained (in 25 μl) 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 20 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml BSA, 0.5 μg of d29 TP-DNA, 20 μM each dCTP, dGTP, dTTP, and [32P]-dATP (1 μCi), the indicated amount of wild-type or mutant d29 TP, and 10 ng of d29 DNA polymerase. After incubation for the indicated time at 30 °C the reactions were stopped by adding EDTA to 10 mM and SDS to 0.1%. Next, the samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. Quantitation of the DNA synthesized in *vitro* was carried out from the amount of radioactivity (Cerenkov radiation) corresponding to the excluded volume. The size of the synthesized DNA was determined by alkaline-agarose gel electrophoresis followed by autoradiography.

**d29 DNA Amplification Assay—** The incubation mixtures contained (in 10 μl) 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 20 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml BSA, 5 ng of d29 TP-DNA, 80 μM each dCTP, dGTP, dTTP, and [32P]-dATP (1 μCi), 8 μg of protein p5 (ssDNA binding protein), 10 μg of protein p6, the indicated amount of wild-type or mutant d29 TP, and 10 ng of d29 DNA polymerase. After incubation for the indicated time at 30 °C, the reactions were stopped and the samples were filtered as described above. The DNA amplification factor was calculated as the ratio between the amount of DNA at the end of the reaction (input d29 DNA plus synthesized DNA) and the amount of input d29 DNA. Quantitation of the DNA synthesized in *vitro* was carried out from the amount of radioactivity (Cerenkov radiation) corresponding to the excluded volume (2.68 nml of dNTP incorporated, which corresponds to 0.93 × 10⁸ rpm, allows the synthesis of 887 ng of dsDNA). The size of the synthesized DNA was determined by alkaline-agarose gel electrophoresis followed by autoradiography. To study the parental TP function of the class I mutants, the reactions were stopped and the samples were filtered as described above.

**RESULTS**

**Design and Construction of Mutant TPs—** Like d29, the genomes of the related *Bacillus* phages φ15, PZA, BS32, B103, M2, NF, and GA-1 contain a linear double-stranded DNA molecule of approximately 20 kilobases with a TP molecule linked to their 5′ ends. Based upon complete or partially known DNA sequences these phages have been classified into three groups (29). The first group includes phages φ29, φ15, PZA, and BS32, the second group includes B103, M2, and NF, and the third group contains GA-1 as its sole member. The DNA sequences of the regions encoding the TP are available for φ29 and PZA of group one, NF and B103 of group two, and GA-1 of group three. These TPs are very similar in size, ranging from 265 to 267 amino acids, sharing an overall similarity of 40.1% (not shown). Analysis of the amino acid sequences of these TPs, using the algorithms of Lupas et al. (16), showed that in all these cases the region located between approximately amino acids 80 and 120 has a high probability to form an α-helical coiled coil structure (results not shown). An alignment of this region is shown in Fig. 2A. Since protein di- or multimerization is often formed through interactions of the hydrophobic sides of amphiatic α-helices (15–17), this conserved region of the TP may be a protein-protein interaction domain.

In this study we analyzed whether the putative coiled coil domain of d29 TP, described above, is involved in recognition of the origin of replication through interaction between the α-helices of the primer and parental TP. For this, we introduced various mutations in the DNA region of the d29 TP encoding the putative coiled coil (see “Materials and Methods”) and studied the behavior of the purified mutant TP proteins in *vitro* d29 DNA replication assays. Fig. 2B shows a helical wheel presentation of the putative coiled coil region and the position of the various mutations introduced. On one hand...
in mutant "4A" the four residues Val84, Ile91, Val105, and Met112 were changed into Ala. Whereas these residues are predicted not to change the ability to form an α-helix, the hydrophobic interactions between the α-helices may be altered. On the other hand, positively charged residues located at the non-hydrophobic side of the α-helix (position f) were replaced by the negatively charged residue glutamic acid. Therefore, with possible intrahelical electrostatic attractions. Thus, in mutant "RKE" the residues Arg103 and Lys110, and in mutant "3E" the residues Arg96, Arg103, and Lys110 were changed into Ala. Whereas these residues are predicted to not change the ability to form an α-helix, the hydrophobic interactions between the α-helices may be altered. On the other hand, positively charged residues located at the non-hydrophobic side of the α-helix (position f) were replaced by the negatively charged residue glutamic acid with the aim to interfere with possible intrahelical electrostatic attractions. Thus, in mutant "RKE" the residues Arg103 and Lys110, and in mutant "3E" the residues Arg96, Arg103, and Lys110 were changed into Ala. Whereas these residues are predicted not to change the ability to form an α-helix, the hydrophobic interactions between the α-helices may be altered. On the other hand, positively charged residues located at the non-hydrophobic side of the α-helix (position f) were replaced by the negatively charged residue glutamic acid.

After site-directed mutagenesis (see "Materials and Methods"), the corresponding DNA fragments were cloned in-frame with the wild-type TP in %.

### Table II

| mutant | TP-DAMP formation* | TP-DNA replicationb |
|--------|-------------------|---------------------|
| wt     | 100               | 100                 |
| Class I|                   |                     |
| VMA    | 153               | 114                 |
| 3E     | 103               | 85                  |
| Class II|                  |                     |
| VIA    | 24                | 32                  |
| RKE    | 30                | 56                  |
| Class III|                 |                     |
| 4A     | <1                | 9                   |

*a Numbers indicate the activity of mutant TPs with respect to the wild-type TP in %.
*b Values correspond to the relative amount of incorporated dAMP.

**Origin of Replication Recognition by Terminal Protein**

Hydrophobic residues predicted to form part of the hydrophobic interface (position α) were changed into alanine residues. Thus, in mutant "VIA" the residues Val48 and Ile51 were changed, in mutant "VMA" residues Val105 and Met112 were changed, and in mutant "4A" the four residues Val48, Ile51, Val105, and Met112 were changed into Ala. Whereas these residues are predicted not to change the ability to form an α-helix, the hydrophobic interactions between the α-helices may be altered. On the other hand, positively charged residues located at the non-hydrophobic side of the α-helix (position f) were replaced by the negatively charged residue glutamic acid with the aim to interfere with possible intrahelical electrostatic attractions. Thus, in mutant "RKE" the residues Arg103 and Lys110, and in mutant "3E" the residues Arg96, Arg103, and Lys110 were changed into glutamic acid, respectively.

Activity of Mutant TPs to Function as Primer for Replica-

**Fig. 3. In vitro protein-primed initiation of φ29 DNA replica-

reaction.** Reaction mixtures contained, in addition of φ29 DNA polymerase (20 ng) and wild-type (wt) or mutant TP (10 ng), 500 ng of φ29 TP-DNA. After incubation for 5 min at 30 °C, the reactions were stopped, processed, and analyzed by SDS-PAGE and autoradiography (see "Materials and Methods" for details). Relative amounts of incorporated [α-32P]-dAMP were calculated by densitometric analysis of the autoradiograph. The various TPs used and the position of TP-dAMP are indicated.

**Activity of Mutant TPs in φ29 DNA Replication—During φ29 DNA replication the formation of the first TP-dAMP is directed by the second nucleotide at the 3'-end of the template.** Then, the TP-dAMP complex slides-back one nucleotide to recover the information of the terminal nucleotide (8). After this step the φ29 DNA polymerase synthesizes a short elongation product before dissociating from the TP (transition step; Ref. 9). It is only after the sliding-back and the transition step that the φ29 DNA polymerase carries out highly processive elongation, which is coupled to strand-displacement, to complete replication of the parental strand. To study possible effects of the mutations introduced in the TP on DNA polymerase elongation, replication assays were carried out in which one full round of replication is allowed (see "Materials and Methods"). The results, presented in Fig. 4 and Table II, show that the classification of the mutant TPs in three groups based upon their activity in initiation reactions can be extended to the replication assays. Thus (i) the amount of full-length φ29 DNA synthesized after 10 min using mutants VMA and 3E was similar to that synthesized using wild-type TP, (ii) those synthesized using mutants VIA and RKE were lower and, (iii) the amount of full-length φ29 DNA synthesized was only 9% compared with the wild-type in case of mutant 4A. Only 0.5% of φ29 DNA was labeled in control reactions in the absence of TP (results not shown). These results suggest that most of the low activity of mutant 4A is due to TP-primed replication.

As shown in Fig. 4, the size of the φ29 DNA products analyzed after 5 min had not reached full-length φ29 DNA indicating that replication had not completed one round of replication at this time. The average length of the φ29 DNA molecules synthesized in assays containing either of the class I or class II mutant TPs was similar to that synthesized in the presence of wild-type TP. These results suggest that the mutations do not affect the elongation rate of the DNA polymerase. The lower
amounts of replicated ϕ29 DNA observed in the assays with the class II mutants is, therefore, probably due to a partial defect in the initiation reaction (see above).

**TP/DNA Polymerase Interaction Is Affected in Mutant TPs VIA, RKE, and 4A**—As described above, the observed lower levels of activity of the class II and class III mutant TPs in the initiation and replication reactions are possibly due to a partial defect in the proper interaction between these TPs and the DNA polymerase. This defect could be a consequence of (i) weaker interactions between TP and DNA polymerase or (ii) the formation of stable but non-functional complexes. To discriminate between these possibilities the various TP mutants were incubated with DNA polymerase to allow heterodimer complex formation after which these were subjected to glycerol gradients (see “Materials and Methods”). In addition to equimolar amounts of TP and DNA polymerase, BSA, which is a monomer in solution, was loaded on top of the gradients to serve as a molecular weight marker. After centrifugation, fractions of the gradients were analyzed by SDS-PAGE. As shown in Fig. 5A, the wild-type TP and DNA polymerase were recovered in the same fractions. Both proteins migrated to positions having an apparent molecular weight slightly higher than that of BSA. These results show that under these conditions the wild-type TP and DNA polymerase migrated as a heterodimer complex in the gradient and, as expected, show that these proteins interact strongly. Similar results were obtained using the class I mutant TPs VMA and 3E, showing that also these TPs form stable heterodimer complexes with the DNA polymerase (results not shown). However, under the same conditions, a small percentage of the class II mutant TPs VIA and RKE (Fig. 5B) and RKE (Fig. 5C) did not migrate to the heterodimer position. In the case of the class III mutant TP 4A, the majority of the two proteins were recovered in separate fractions (Fig. 5D), each migrating to positions corresponding to their monomer state (as determined in separate gradients, not shown). Together, these results suggest that the interactions between DNA polymerase and mutant TPs VIA and RKE are moderately affected and those with mutant 4A are strongly affected.

**Efficiency of the Class II Mutant TPs, but Not the Class III Mutant TP, in Initiation and Replication Assays Are Enhanced to Nearly Wild-type Levels by Increasing Their Concentration in the Reaction Mixtures**—The results presented above showed that the activity of the mutant TPs belonging to class II (VIA and RKE) and class III (4A) are moderately and severely affected, respectively, in the initiation and replication assays. The decreased activities of the class II mutant TPs are likely due to a partial defect in the interaction between these TPs and the DNA polymerase. Diminished TP-DNA polymerase interactions, resulting in decreased initiation and replication activity, have been observed with some TPs containing mutations in the RGD motif (position 256–258). The interactions of these mutant TPs with the DNA polymerase could be restored to almost wild-type levels by increasing the concentration of the TP (31). To study whether the activity of the class II and III mutant TPs could be restored similarly, their functional activity in initiation and replication assays was analyzed using reaction mixtures containing increasing concentration of either of these mutant TPs. Assays with wild-type TP in the reaction mixtures were included to serve as internal controls. Increasing concentrations of mutant 4A in the reaction mixtures resulted only in a very slight stimulation of the initiation (Figs. 6, A and B) and replication (Fig. 6C) reactions. However, whereas initiation reactions were moderately stimulated at increasing concentrations of wild-type TP, these conditions highly stimulated the initiation reactions in the case of the class II mutant TPs VIA and RKE, both in the absence (Fig. 6A) and presence (Fig. 6B) of TP-DNA. A highly stimulating effect over an increasing TP range was also observed for these mutants in replication reactions (Fig. 6C). The amount of synthesized ϕ29 DNA was slightly inhibited at the highest TP concentration analyzed (80 ng). Free TP binds nonspecifically to DNA and these conditions have been shown to inhibit the synthesis of ϕ29 DNA in replication assays (32, 33). Therefore, binding of the excess of TP to the template DNA probably caused the inhibition observed at high TP concentrations.

Together, these results show that the efficiency in the initiation and replication assays of class II mutant TPs (VIA and RKE), caused by a moderate defect in TP-DNA polymerase interaction, can be restored to wild-type levels by increasing the concentration of these TPs in the reaction mixture. However, the severe TP-DNA polymerase interaction defect of the class III mutant (4A) could not be restored to wild-type levels.
by increasing its concentration in the reaction mixtures.

Class I and Class II Mutant TPs Are Affected in Their Parental TP Function—The results described above showed that the class I mutant TPs (VMA and 3E) and the class II mutant TPs (VIA and RKE), the latter at an increased TP concentration, behaved essentially as wild-type TP in replication assays. This indicates that the putative interaction between these mutant TPs with the wild-type parental TP is not affected. However, since the replication assays carried out only give rise to one round of replication (14), possible effects of these mutant TPs on its function as parental TP are not assessed by this method.

An in vitro \( \phi 29 \) DNA amplification system has been described that requires, in addition to TP-DNA, the following purified \( \phi 29 \)-encoded proteins: TP, DNA polymerase, the dsDNA-binding protein p6, and the ssDNA-binding protein p5 (34). In these amplification reactions, up to 10 rounds of replication were achieved. Thus, under these conditions, primer TP that becomes covalently attached to the newly synthesized DNA strand during the first round of replication will act as parental TP in subsequent replication rounds. Hence, we exploited this system to study the function of the class I (VMA and 3E) and class II (VIA and RKE) mutants as parental TP. Due to the low initiation activities, the class III mutant (4A) was not studied in amplification assays. Standard conditions (see “Materials and Methods”) were used in the amplification assays containing wild-type or class I mutant TPs. The concentration of the class II mutant TPs in the reaction mixtures was increased 4-fold with respect to the standard conditions. This modification was introduced because these conditions were required to obtain wild-type levels with these mutant TPs in the replication assays (see above). Because the higher level of TP may affect the amplification reaction due to binding of the free TP to the template DNA (note that far less template DNA is used in amplification compared with replication assays), amplification levels of class II mutant TPs were compared with those obtained in assays containing a 4-fold excess of wild-type TP. The synthesis of DNA during the amplification reaction was studied as a function of time. The various samples were first used to measure the amount of incorporated \([\alpha-\text{32P}]\text{dAMP}\) after which they were subjected to alkaline-agarose gel electrophoresis. The amounts of de novo synthesized DNA (in nanograms) was quantified from the amount of radioactivity incorporated into DNA, and amplification factors (see Table III) were calculated as the ratio between the amount of DNA synthesized (input DNA plus synthesized DNA) and the amount of input DNA.

## Table III

| \( \phi 29 \) TP | Incubation time |
|----------------|----------------|
|                | 5 min | 10 min | 20 min | 40 min | 80 min | 160 min |
| Class I        |       |        |        |        |        |         |
| wt (5 ng)      | 1.3   | 1.9    | 4.1    | 20     | 58     | 61      |
| VMA (5 ng)     | 1.2   | 1.3    | 1.4    | 1.7    | 2.1    | 2.8     |
| 3E (5 ng)      | 1.1   | 1.2    | 1.2    | 1.4    | 1.7    | 2.0     |
| Class II       |       |        |        |        |        |         |
| wt (20 ng)     | 1.3   | 1.8    | 5.3    | 4.7    | 75     | 79      |
| VIA (20 ng)    | 1.1   | 1.2    | 1.3    | 1.4    | 1.5    | 1.7     |
| RKE (20 ng)    | 1.1   | 1.3    | 1.6    | 2.4    | 4.0    | 13      |

The results with the wild-type TP and the class I mutant TPs (standard amounts of TP) are presented in Fig. 7, and those obtained with the wild-type TP and class II mutant TPs (4-fold excess of TP) are presented in Fig. 8. The amount of DNA synthesized in the presence of wild-type TP (both, with equimolar or 4-fold excess of TP relative to DNA polymerase) increased with time (Figs. 7 and 8, left frames). Fig. 7 shows that the amount of DNA synthesized after 5 and 10 min in the presence of either of the class I mutant TPs was only slightly less compared with those synthesized by the wild-type TP (see also Table III). As in the replication assays, full-length \( \phi 29 \) DNA was observed after 10 min with the mutant TPs and it happens with the wild-type TP. These results show that, also under these conditions, the putative interaction between these mutant primer TPs with the wild-type parental TP is not affected. However, compared with the wild-type TP, far less DNA was synthesized with these mutant TPs at later times. After 160 min the input DNA was amplified only with a factor of 2.8 (mutant VMA) and 2.0 (mutant 3E) versus 61 with the wild-type TP (see Table III). Even after longer incubation times the amplification factor in assays containing either of these mutant TPs never exceeded a value of 3 (results not shown).

Similar results were obtained with the class II mutant TP VIA (see Fig. 8). Also in this case full-length \( \phi 29 \) DNA was observed after 10 min. Likewise, whereas DNA synthesis continued efficiently with the wild-type TP, reaching an amplification level of 79 after 160 min, the DNA was only amplified by a factor of 1.7 in case of the mutant VIA (see Table III). The other class II mutant (RKE), however, showed an intermediate phenotype. Although the level of amplification was lower com-
TP-DNA as template, 5 ng of wild-type or mutant TPs, and 10 ng of DNA polymerase. The amplification assays were carried out as described under “Materials and Methods” in the presence of 5 ng of \( \Phi 29 \) TP-DNA as template, 5 ng of wild-type or mutant TPs, and 10 ng of \( \Phi 29 \) DNA polymerase. After incubation for the indicated times at 30 °C, the reaction was stopped and quantitated as the total amount (in nanograms) of dNTP incorporated. After quantitation the samples were used to determine the size of the synthesized DNAs by subjecting the samples to alkaline-agarose gel electrophoresis (A). The migration position of unit length \( \Phi 29 \) DNA is indicated. wt, wild-type; bp, base pairs. The quantitative data are graphically represented in B.

One round of replication of all the input template DNA molecules will lead to an amplification factor of 2 and after completion of a second round of replication the amplification factor will be 4. Thus, DNA synthesis appears to have stalled before completing the second round of replication when the reaction mixtures contained either of the class I mutant TPs or the class II mutant TP VIA. The template DNA that is used in the amplification reactions contains a wild-type TP molecule at each DNA end (origin). Each of the two daughter molecules that are generated after one round of replication in amplification assays carried out with mutant TP in the reaction mixture will contain one origin with a wild-type TP and the other with a mutant TP (see Fig. 1 for a schematic overview). Inactivation of the origin of replication upon incorporation of a mutant TP may explain the results obtained. In that case, only the wild-type TP containing origins would be active in the second round of replication resulting in a maximum amplification factor of 3. Both origins of the DNA molecules generated after this second incomplete round of replication will contain a mutant TP, and, according to the model, further replication would cease. This view is in agreement with the data obtained in quantitation of the DNA synthesized in vitro (see Table III). Thus, the results strongly suggest that the mutations present in the class I mutants and in the class II mutant TP VIA result in a failure of these mutants to function as parental TPs. In addition, the results indicate that the mutations present in class II mutant RKE affect the parental TP function, although they do not fully inhibit this function.

Addition of Wild-type TP/DNA Polymerase Heterodimer Re-

stores DNA Amplification—The above results showed that mutations in the class I TPs and in the class II TP VIA inactivate its parental TP function. Most likely, the mutations prevent interactions between the mutant parental TP and mutant primer TP or they lead to non-functional interactions. The results of the amplification assays do not allow to discriminate between these two possibilities. In addition, the amplification assays do not allow to discriminate whether the presence of a mutant TP at the DNA end inactivates this origin because it excludes (functional) interactions with (i) only the mutant primer TP or (ii) with either a wild-type TP or a mutant primer TP (dominant effect).

In order to discriminate between these possibilities the following experiments were carried out. After amplification for 80 min, the time at which replication with either of these three mutant TPs had nearly stopped, extra TP/DNA polymerase heterodimers were added to the amplification reactions (Fig. 9, see “Materials and Methods” for details). In the case of the class I mutant TP 3E (Fig. 9, left frame), renewed incorporation of dAMP was observed upon the addition of wild-type TP/DNA polymerase heterodimer. Contrary, addition of 3E TP/DNA polymerase heterodimers did not result in renewed dAMP incorporation. These results show that the parental 3E TP forms functional interactions with wild-type TP/DNA polymerase heterodimer but not with a heterodimer containing the 3E mutant TP. Therefore, the mutations in 3E are critical when they are present in both the primer and parental TP, most likely, affecting their proper interaction required for a functional initiation reaction. In addition, the renewed dAMP incorporation suggests that the mutant TP-containing origin is
not blocked by a non-functional interaction with mutant TP/DNA polymerase heterodimer.

Also in the case of the other class I mutant TP (VMA) and in case of the class II mutant TP VIA (using a 4-fold excess of wild-type or mutant TP, see "Materials and Methods") the mutant TP-containing origins were rescued upon addition of wild-type TP/DNA polymerase heterodimer (Fig. 9, middle and right frame, respectively). Although low levels of DAMP incorporations were observed upon the addition of extra mutant TP/DNA polymerase heterodimer, the calculated amplification factors did not exceed a value of three. Together, these results indicate that inactivation of the origin is caused by the lack of functional interactions between mutant primer and mutant parental TP.

**DISCUSSION**

In this work we have introduced mutations in the region of the φ29 TP constituting a putative coiled coil region and analyzed the corresponding purified proteins for possible effects of these mutations on its function using *in vitro* φ29 DNA initiation, replication, and amplification assays. The principal conclusion drawn from the results obtained is that the putative coiled coil region of TP is a main determinant involved in recognition of origin of replication, the first step in initiation of φ29 DNA replication.

The linear φ29 genome uses a protein-primed DNA replication mechanism. The φ29 DNA ends, containing a covalently attached TP molecule, constitute the origins of replication. The first step of initiation of DNA replication is the recognition of these origins by a TP/DNA polymerase heterodimer. The TP molecule in the heterodimer functions as primer for the subsequent replication initiation step. Blunt-ended DNA fragments containing the left or right φ29 DNA ends, but not internal φ29 DNA fragments, were active as templates in *in vitro* initiation reactions, indicating that specific DNA sequences located at the φ29 DNA ends are involved in origin recognition (13, 35, 36). However, the activity of an excess of φ29 DNA ends lacking a parental TP is only about 10% compared with limited amounts of DNA ends containing a parental TP (13, 36). These latter results show that the parental TP is the major signal in the template for origin recognition and strongly suggest that the TP/DNA polymerase heterodimer is recruited to the origin through interaction with the parental TP. Parental TP molecules can interact with each other (37, 38) which may suggest that recruitment of the heterodimer to the origin is brought about by protein-protein contacts between the parental and primer TP. Here, we describe the detection of a putative coiled coil region between residues 84 and 118 of the TP sequence, that could be involved in interactions between parental and primer TP and, thereby, being important for origin recognition.

To test this hypothesis, various mutations were introduced in the TP and functional analyses of the purified mutants were carried out. Initiation (with and without template DNA) and replication assays showed that the mutant TPs could be grouped into three classes. The class I mutant TPs (VMA and 3E) had similar activities compared with the wild-type TP in initiation and replication assays, indicating that they interact correctly with the DNA polymerase and that they function well as primer TP. The class II mutants (VIA and RKE) were shown to be moderately affected in their interaction with the DNA polymerase. However, by increasing the concentration of these mutant TPs in the reaction mixtures near wild-type levels of activities were obtained in initiation and replication assays. In addition, the mutations present in the class I and class II mutant TPs did not affect the elongation rate. Moreover, the wild-type activities observed in the initiation and replication assays with the mutant TPs of class I and class II (using a TP excess), indicate that these mutant TPs interact correctly with the wild-type parental TP.

A clear difference, however, was observed with these mutant TPs in the amplification assays. Whereas the input DNA was amplified 61- (equimolar amounts of DNA polymerase and primer TP) and 79-fold (4-fold excess of primer TP) in assays with wild-type TP, much lower amplification levels were observed in the amplification assays with the mutant TPs of class I and class II (using a TP excess), indicating that these mutant TPs interact correctly with the wild-type parental TP.
mutant TPs. Thus, the mutations in the class I TPs and in the class II TP VIA would specifically affect the parental TP function. The observation that amplification was restored upon addition of wild-type TP/DNA polymerase heterodimers but not upon addition of mutant TP/DNA polymerase heterodimers show that the mutations in these TPs are only critical when they are simultaneously present in both the primer and the parental TP. Most likely, the mutations impede functional interactions between these mutant parental and mutant primer TPs.

Importantly, the latter results also demonstrate that these mutant parental TPs make functional interactions with wild-type primer TP and, therefore, exclude the possibility that the mutations have affected the overall structure of the parental TP that might cause inactivation of the origin. Rather, these results support the view that the defect of the parental TP function of these mutants is due to a failure of functional interactions between the amphipatic α-helices of mutant primer and mutant parental TP.

The class II mutant TP RKE displayed an intermediate phenotype in the amplification assays. Whereas the amplification factors were comparable to those obtained with the wild-type TP up to 10 min they were lower at later times. Nevertheless, after 160 min an amplification factor of 13 was reached with this mutant TP. This shows that replication can surpass the second round of replication which indicates that, although less efficient as compared with the wild-type TP, functional interactions occur between this mutant primer and mutant parental TP.

Surprisingly, whereas the TP mutant RKE, containing two amino acid substitutions, was moderately affected in its interaction with the DNA polymerase, the TP mutant 3E, containing an extra amino acid substitution (R96E), in mutant 3E compensates the observed moderate DNA polymerase interaction defect of RKE. Interestingly, however, of these two mutant TPs only the mutant RKE was able to surpass the second round of replication in amplification assays. Also after the addition of extra 3E TP/DNA polymerase the amplification reaction was unable to pass the second round of amplification (see Fig. 9). These results indicate that the additional mutation in mutant 3E (R96E) is responsible for the complete inactivation of its parental TP function.

TP mutant 4A (class III) was shown to be severely affected in initiation reactions, originally caused by a severe defect in the interaction of this mutant with the DNA polymerase, as assessed by glycerol gradient analysis. These results may suggest that the amphipatic α-helix is involved in interaction with the DNA polymerase. However, it is also possible that the quadruplet mutation in 4A affects the protein structure to such an extent that correct interaction with the DNA polymerase is impaired. Because of its low activity in initiation, we were unable to assess possible effects of this mutant on the parental TP function.

Several amino acid residues located in the N-terminal part of the φ29 TP that are highly conserved among TPs encoded by various phages were mutated and the purified mutants were analyzed for their functionality (14). Three of these mutant proteins showed a similar phenotype to that of the class I mutant TPs analyzed in this study. Thus, also these three mutants behaved as wild-type TP in initiation and replication assays, but replication was stalled in the amplification assays before the second round was completed. The mutations giving rise to this phenotype are located at positions 80 (N80S) and 82 (Y82S and Y82L) of the φ29 TP. The results obtained with these mutant TPs led to the conclusion that residues Asn80 and Tyr29 of φ29 TP are essential for functional interactions between primer and parental TP. Interestingly, these mutations are located just before the predicted amphipatic α-helix. Taking into account the results presented in this work, we consider it also possible that the mutations introduced at positions 80 and 82 of the φ29 TP may affect the orientation of the downstream located amphipatic α-helix underlying the observed failure of interaction between these mutant primer and mutant parental TPs. Recently, evidence has been obtained that, in addition to the primer TP-parental TP interactions, recognition of the origin of replication of φ29 DNA also involves interactions between the DNA polymerase and the parental TP (39).

In summary, the results presented in this work strongly suggest that interactions between the amphipatic α-helices of φ29 primer and parental TP are important for recognition of the φ29 origin of replication and that this process, most likely, also involves interactions between the parental TP and the DNA polymerase (39). Confirmation of these results may come from the determination of the three-dimensional structure of the DNA polymerase-TP complex that will elucidate the position of amphipatic α-helix of TP in this complex. In addition, this crystal structure would provide an excellent tool to select specific amino acid residues of the TP and/or DNA polymerase that may be important for origin recognition. Site-directed mutagenesis of these residues would lead to further insight of this process.

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REFERENCES

1. Kornberg, A., and Baker, T. A. (1992) DNA Replication, W. H. Freeman, San Francisco.
2. Dutta, A., and Bell, S. P. (1997) Annu. Rev. Cell Dev. Biol. 13, 293–332.
3. Salas, M. (1991) Annu. Rev. Biochem. 60, 39–71.
4. Salas, M., Millar, J. T., Leis, J., and DePamphilis, M. L. (1996) in DNA Replication in Eukaryotic Cells (DePamphilis, M. L., ed) pp. 131–176, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
5. Serrano, M., Salas, M., and Hermoso, J. M. (1983) Trends Biochem. Sci. 18, 192–206.
6. Blanco, L., and Salas, M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5325–5329.
7. Hermoso, J. M., Méndez, E., Soriano, F., and Salas, M. (1985) Nucleic Acids Res. 13, 7715–7728.
8. Méndez, J., Blanco, L. Esteban, J. A., Bernad, A., and Salas, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9579–9583.
9. Méndez, J. Blanco, L., and Salas, M. (1997) EMBO J. 16, 2519–2527.
10. Iniciarte, M. R., Salas, M., and Sogo, J. M. (1990) J. Virol. 64, 187–190.
11. Gutiérrez, C., Sogo, J. M., and Salas, M. (1991) Mol. Biol. 222, 983–994.
12. Blanco, L. Prieto, I. Gutiérrez, J., Bernad, A., Lázaro, J. M., Hermoso, J. M., and Salas, M. (1987) J. Virol. 61, 3893–3891.
13. Gutiérrez, J. Vinios, J. Prieto, I. Méndez, E. Hermoso, J. M., and Salas, M. (1986) Virology 155, 474–483.
14. Illana, B., Lázaro, J. M., Gutiérrez, C., Meijer, W. J. J., Blanco, L., and Salas, M. (1990) J. Biol. Chem. 265, 15073–15079.
15. Cohen, C., and Parry, D. A. (1994) Science 263, 488–489.
16. Lupas, A. van Dyke, M., and Stock, J. (1991) Science 252, 1162–1164.
17. Lupas, A. (1986) Trends Biochem. Sci. 21, 375–382.
18. Hodges, R. S. (1992) Cell. Biol. 122, 122–124.
19. Hu, J. C., O’Shea, E. K., Kim, P. S., and Sauer, R. T. (1990) Science 250, 1400–1403.
20. Zhou, N. E., Hay, C. M., and Hodges, R. S. (1992) J. Biol. Chem. 267, 2664–2670.
21. Zhou, N. E., Hay, C. M., and Hodges, R. S. (1992) Biochemistry 31, 5739–5746.
22. Kuo, W. D., Minera, O. A., Kay, C. M., and Hodges, R. S. (1985) J. Biol. Chem. 260, 25495–25506.
23. Yanish-Perron, C., Vieira, J., and Messing, J. (1985) Gene 33, 103–119.
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. Penálv, M. A., and Salas, M. (1982) Proc. Natl. Acad. Sci. U. S. A. 89, 15073–15079.
26. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.
27. Innis, M. A., and Gelfand, D. H. (1990) in PCR Protocols: A Guide to Methods (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds) Academic Press, New York.
28. Schon, U. and Schumann, W. (1994) Gene (Amst.) 147, 91–94.
29. Peceneková, T., and Paces, V. (1999) in Mol. Biol. 48, 197–208.
30. Blanco, L., Bernad, A., Esteban, J. A., and Salas, M. (1992) J. Biol. Chem. \textbf{267}, 1225–1230
31. Illana, B., Zaballos, A., Blanco, L., and Salas, M. (1998) Virology \textbf{248}, 12–19
32. Garmendia, C., Salas, M., and Hermoso, J. M. (1988) Nucleic Acids Res. \textbf{16}, 5727–5740
33. Zaballos, A., and Salas, M. (1989) Nucleic Acids Res. \textbf{17}, 10353–10366
34. Blanco, L., Lázaro, J. M., De Vega, M., Bonnin, A., and Salas, M. (1994) Proc. Natl. Acad. Sci. U. S. A. \textbf{91}, 12198–12202
35. García, J. A., Peñalva, M. A., Blanco, L., and Salas, M. (1984) Proc. Natl. Acad. Sci. U. S. A. \textbf{81}, 80–84
36. Gutiérrez, J., García, J. A., Blanco, L., and Salas, M. (1986) Gene (Amst.) \textbf{43}, 1–11
37. Ortíz, J., Viñuela, E., Salas, M., and Vasquez, C. (1971) Nat. New Biol. \textbf{234}, 275–277
38. Salas, M., Mellado, R. P., Viñuela, E., and Sogo, J. M. (1978) J. Mol. Biol. \textbf{119}, 269–291
39. González-Huici, V., Lázaro, J. M., Salas, M., and Hermoso, J. M. (2000) J. Biol. Chem. \textbf{275}, 14678–14683
The Putative Coiled Coil Domain of the φ29 Terminal Protein Is a Major Determinant Involved in Recognition of the Origin of Replication
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