
Site-directed mutagenesis in the DNA linking site of bacteriophage ϕ 29 terminal protein: isolation and characterization of a Ser₂₃₂ → Thr mutant

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ABSTRACT

By site-directed mutagenesis we have changed the serine residue 232 of the ϕ 29 terminal protein, involved in the covalent linkage to dAMP for the initiation of replication, into a threonine residue. The mutant terminal protein has been purified to homogeneity and shown to be inactive in the formation of the initiation complex; nevertheless, the mutant protein retains its ability to interact with the ϕ 29 DNA polymerase and with the DNA. The results obtained indicate a high specificity in the linking site of the terminal protein.

INTRODUCTION

B. subtilis phage ϕ 29 has a linear, double-stranded DNA of 19,285 base pairs (1-3) with a viral terminal protein, p3, covalently linked to the two 5'ends through a phosphodiester bond between the OH group of serine residue 232 and dAMP (4). The viral genome replicates by a strand-displacement mechanism starting from either end (reviewed in 5), in which the free terminal protein plays a crucial role priming the initiation reaction by formation of a covalent complex with 5'dAMP catalyzed by the viral DNA polymerase p2 (6,7). An in vitro system has been developed for the formation of the initiation complex in which purified proteins p2 and p3, and ϕ 29 DNA-protein p3 complex as template are the only macromolecular requirements (6,7). Furthermore, in this system the initiation complex can be elongated to full-length ϕ 29 DNA (8). Addition of NH_4^+ ions and viral protein p6 to the in vitro system stimulate ϕ 29 DNA replication (8-11).

The formation of the initiation complex p3-dAMP is accomplished by interaction of a free molecule of the terminal protein with the viral DNA polymerase (11) and the recognition of the origins of replication, mainly by interaction with the terminal

protein covalently attached to the 5' ends of the template. Initiation can also occur in a lesser extent with protein-free templates containing at least the terminal 12 base pairs of either ϕ 29 DNA end (12-14).

By site-directed mutagenesis we are changing different amino acid residues in the terminal protein to correlate the structure with the function of the protein, by studying the formation of the initiation complex, and the interaction with the DNA polymerase and the DNA. In this paper we address the question of the specificity of the linking site by changing the serine residue 232, the donor of the OH group in the phosphodiester linkage with the first nucleotide, by a threonine residue, that would be the minimal structural replacement able to form that linkage. In fact, the *Streptococcus pneumoniae* phage Cp-1 terminal protein is linked to the DNA through a phosphothreonine bond (15). We show here that the Ser₂₃₂→Thr change completely abolishes the priming function of the ϕ 29 terminal protein.

MATERIALS AND METHODS

Plasmids, bacteria and phages

Restriction fragments containing the ϕ 29 gene 3 truncated at the carboxyl end were obtained from plasmid pRMT121 (16). The complete gene was obtained from plasmid pRMn25 (17) and the gene 3 expression vector was derived from plasmid pPLc28 (18), obtained from E. Remaut.

E. coli strain Δ lacpro, supE, thi, strA, sbcB15, endA, hspR4, F' traD36, proAB, lacI^QZ Δ M15, also called JM103 (19) was a gift from Antibióticos S.A. and used as a host of M13-derived vectors, and *E. coli* strain M72 Sm^RlacZam Δ bio-uvrB Δ trpEA2 (λ Nam7 Nam53cI857 Δ H1), here designated K-12 Δ H1 Δ trp (20), was obtained from M. Zabeau and used as host of expression vectors derived from pPLc28.

Phage M13mp8 replicative form (21) used to perform the site-directed mutagenesis was a gift from Antibióticos S.A.

Enzymes and chemicals

Restriction enzymes, T4 polynucleotide ligase and Klenow fragment of *E. coli* DNA polymerase I were from Boehringer Mannheim, IPTG and X-gal from Sigma, deoxynucleoside triphosphate and dideoxynucleoside triphosphates from PL Biochemicals.

Sequencing primer, ^{35}S -methionine (1000 Ci/mmol), [γ - ^{32}P]ATP (~3000 Ci/mmol), [^{35}S]dATP α S (~600 Ci/mmol) and [α - ^{32}P] dATP (~410 Ci/mmol) were from Amersham International, plc. The synthetic oligonucleotide used in the site-directed mutagenesis was a gift from Antibióticos S.A., and further purified by gel filtration through a Sephadex G-50 column and electrophoresis in a 15% polyacrylamide gel containing 8.3 M urea. Prior to use, the oligonucleotide was phosphorylated with T4 polynucleotide kinase as described (22) and sequenced by the Maxam and Gilbert method (23).

DNA manipulations

Plasmids were isolated as described (24) except for analytical purposes where the alkaline lysis method (25) was followed. Restriction enzymes were used as recommended by the supplier. Ligation of restriction fragments, filling-in of cohesive ends with the Klenow fragment, and analytical agarose gel electrophoresis were as described (26). Purification of restriction fragments was done by electrophoresis in low melting agarose gels (27). *E. coli* K-12 Δ H1 Δ trp cells were made competent and transformed as described (28).

Site-directed mutagenesis in phage ϕ 29 gene 3

Plasmid pRmt121 (16), derived from pPLc28 (18), contains the ϕ 29 DNA HindIII G fragment, coding for protein p3, except for the last five amino acids at the carboxyl end. The EcoRI-BamHI fragment, containing the ϕ 29 HindIII G fragment, was isolated and inserted into the corresponding targets of the polycloning site of the replicative form of phage M13mp8. *E. coli* JM103 cells were transformed, and single-stranded DNA from white plaques was used as template for site-directed mutagenesis performed by the method of Zoller and Smith (22). One pmol of single-stranded DNA was hybridized with 30 molar excess of a 18-mer synthetic oligonucleotide complementary to the gene 3 region containing the Ser₂₃₂ codon AGT in a central position, with a G:G mismatch to give rise to the threonine codon ACT. After primer extension, the covalently closed circular DNA was isolated in a 5 to 20% linear sucrose gradient in 1 M NaCl, 0.2 M NaOH and 2 mM EDTA, centrifuged at 100,000 x g during 100 min and used directly to transform *E. coli* JM103 cells. Phage obtained from 46 plaques was used directly in a dot-blot test (22). Two putative mutants

were grown in E. coli JM103 cells, replated and the dot-blot test was performed once again with phage from isolated plaques. The presence of the mutation was confirmed by sequencing by the chain termination method with [³⁵S]dATP (29) using an universal primer. The recombinant phage was named M13mp8.3T₂₃₂ΔC.

Purification of mutant Ser₂₃₂→Thr of the Ø29 terminal protein

Mutant terminal protein was purified from E. coli K-12ΔH1Δtrp cells transformed with plasmid p3T₂₃₂. The cells were grown at 30°C until mid-log phase, the temperature was raised to 42°C to allow induction and the cells were further grown for two hours. The purification procedure was based in the method described for the wild-type protein (30). Cell extract was obtained by grinding 12 g of cells with alumina and resuspension with 60 ml of buffer A (20 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM EDTA, 7 mM β-mercaptoethanol and 5% glycerol). Alumina and debris were removed by centrifugation for 10 min at 20,000 x g. The extract was passed through a DEAE-cellulose column (100 ml) equilibrated in buffer A containing 0.3 M NaCl, washed thoroughly with the same buffer and protein p3 was eluted with buffer A containing 0.7 M NaCl. Contaminant nucleic acids were removed by precipitation with 0.2% polyethyleneimine in the presence of 1 M NaCl. The supernatant was precipitated with 65% (NH₄)₂ SO₄ in the presence of bovine serum albumin as carrier and the pellet was resuspended in 2 ml of 20 mM Tris-HCl, pH 7.5, 20% glycerol and passed through a phosphocellulose column (0.4 ml) equilibrated in buffer B (20 mM Tris-HCl, pH 7.5, 0.25 M NaCl), washed with the same buffer and protein p3 was eluted with buffer B containing 1 M NaCl and 0.002% Nonidet P-40. The sample was diluted up to 50 mM NaCl with buffer A without NaCl, passed through a second DEAE-cellulose column (0.5 ml) equilibrated in buffer A containing 50 mM NaCl and washed with the same buffer; protein p3 was eluted with buffer A containing 0.1 M NaCl, and concentrated in a phosphocellulose column (0.1 ml) equilibrated in buffer B, and eluted with buffer B containing 1 M NaCl. Mutant protein p3 along the purification steps was identified by radioimmunoassay (30) using antibody against wild-type protein p3. The protein in the different steps was analyzed by SDS-polyacrylamide gel electrophoresis as described (30). The purified protein did not contain contaminating nucleases.

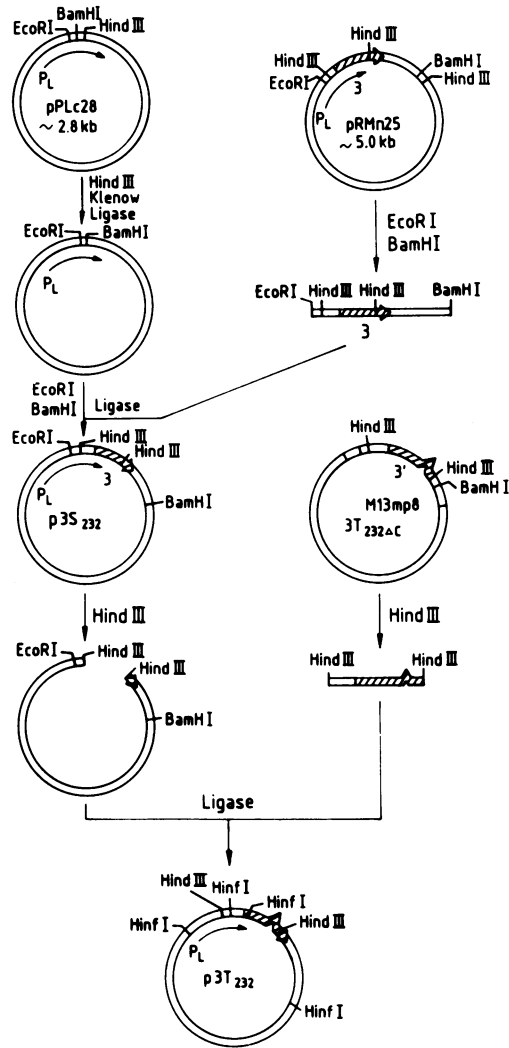


Figure 1. Construction of a recombinant expression plasmid containing the mutation Ser₂₃₂→Thr in ϕ 29 gene 3. The EcoRI-BamHI fragment from plasmid pRMn25, containing ϕ 29 gene 3, was inserted in the expression vector pLc28 after removal of the HindIII restriction site, giving rise to plasmid p3S₂₃₂, in which the ϕ 29 gene 3 is transcribed under the control of the phage λ P_L promoter. The direction of transcription is indicated by the arrow. The HindIII fragment from plasmid p3S₂₃₂, containing gene 3 with a deletion at the carboxyl terminus, was replaced by the corresponding fragment from the replicative form of phage M13mp8.3T₂₃₂ΔC, that contains the mutation Ser₂₃₂→Thr obtained by site-directed mutagenesis. gene 3, gene 3 with the mutation Ser₂₃₂→Thr; the arrow head indicates the carboxyl end.

Assay for the formation of the initiation complex

The reaction mixture contained, in 25 μ l, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1 mM spermidine, 20 mM (NH₄)₂SO₄, 0.25 μ M [α -³²P]dATP (2 μ Ci), 0.5 μ g of ϕ 29 DNA-protein p3 template (31), 19 ng of purified protein p2 (6), 2 μ g of purified protein p6 (9) and 15 ng of purified wild-type (30) or mutant protein p3. After incubation at 30°C for the indicated times, the reaction was stopped by addition of EDTA to 12.5 mM and heating for 10 min at 68°C and the samples were processed as described (10).

RESULTS AND DISCUSSION

Expression of the Ser₂₃₂→Thr mutant of bacteriophage ϕ 29 terminal protein

The strategy followed to clone the mutant gene 3 in an expression vector to overproduce the protein is shown in Fig. 1. The EcoRI-BamHI fragment from plasmid pRMn25 (17) containing ϕ 29 gene 3 was inserted under the control of the λ P_L promoter in the expression vector pPLc28 (18) in which the unique HindIII site was removed by digestion with HindIII and ligation after filling-in the cohesive ends. By transformation of *E. coli* K-12 Δ H1 Δ trp cells, plasmid p3S₂₃₂ was obtained. The ϕ 29 HindIII G fragment containing the mutation was obtained from the replicative form of M13mp8.3T₂₃₂ Δ C (see Materials and Methods), and used to replace the corresponding wild-type fragment from plasmid p3S₂₃₂. The recombinant plasmid containing the mutant gene 3 in the correct orientation was named p3T₂₃₂. Figure 2 shows the presence of the G→C transversion in the HindIII fragment from plasmid p3T₂₃₂ inserted in the replicative form of M13mp8 and sequenced as indicated before (29).

Synthesis of ³⁵S-labelled protein p3 by *E. coli* K-12 Δ H1 Δ trp cells harbouring plasmids p3S₂₃₂ and p3T₂₃₂ is shown in Figure 3. A protein band with the same electrophoretic mobility as the ϕ 29 terminal protein appeared in cells transformed by plasmids p3S₂₃₂ or p3T₂₃₂ after 1 h or 2 h of induction at 42°C, but not in the cells kept at 30°C. This protein was synthesized to a similar extent at least after 3 h of induction. Two other polypeptides were induced; one of them corresponds by its electrophoretical mobility to the viral protein p4 encoded in the cloned HindIII G

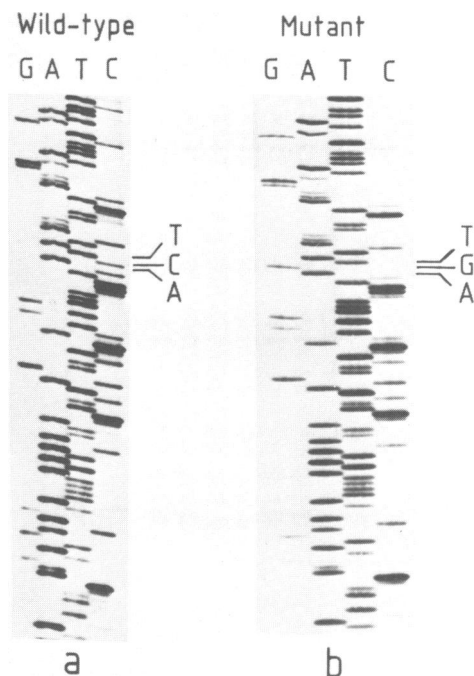


Figure 2. Sequence of the Ser→Thr mutant at position 232 of the $\phi 29$ terminal protein. The HindIII fragment from plasmid p3S₂₃₂' containing the truncated wild-type gene 3, or plasmid p3T₂₃₂' with the truncated mutant gene 3 (see Fig. 1), were cloned in the replicative form of phage M13mp8 and sequenced by the chain termination method (29). The sequence shows the complementary strand of the Ser codon AGT in the wild-type (a) and of the corresponding Thr codon ACT in the mutant (b) gene 3.15.

fragment (16); the other one could be also encoded by the same fragment and affected by the mutation since there is a different electrophoretic mobility between the wild-type and the mutant peptides (Fig. 3 lanes c and d, g and h). These two polypeptides also appear in induced cells containing plasmid pRMn25 (17).

Extracts were prepared from *E. coli* K-12 Δ H1 Δ trp cells containing plasmid p3T₂₃₂ or p3S₂₃₂', and the presence of protein p3 detected by radioimmunoassay; no difference was observed between them (not shown). The extracts were also assayed for their activity in the formation of the initiation complex by complementation with purified $\phi 29$ DNA polymerase, purified protein p6 and $\phi 29$ DNA-protein p3 as template. Formation of p3-dAMP ini-

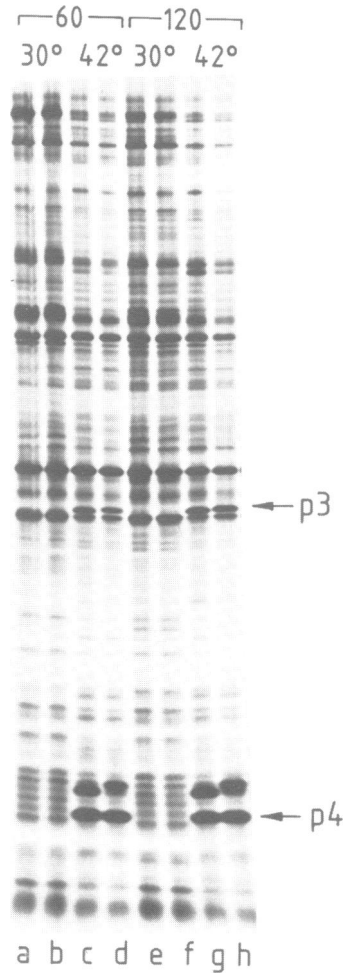


Figure 3. Induced synthesis of the wild-type terminal protein or the Ser₂₃₂→Thr mutant by plasmids p3S₂₃₂ and p3T₂₃₂. Bacterial cultures harbouring plasmids p3S₂₃₂ (lanes a, c, e and g) or p3T₂₃₂ (lanes b, d, f and h) were grown in L broth at 30°C until mid-log phase and the cells were harvested and resuspended in an equal volume of minimal medium; half of the cultures were further grown at 30°C (lanes a, b, e and f) and the other half at 42°C (lanes c, d, g and h) for 60 min (a-d) or 120 min (lanes e-h), labelled with ³⁵S-methionine for 10 min and subjected to SDS-polyacrylamide gel electrophoresis as described (16). The mobility of proteins p3 and p4 are indicated.

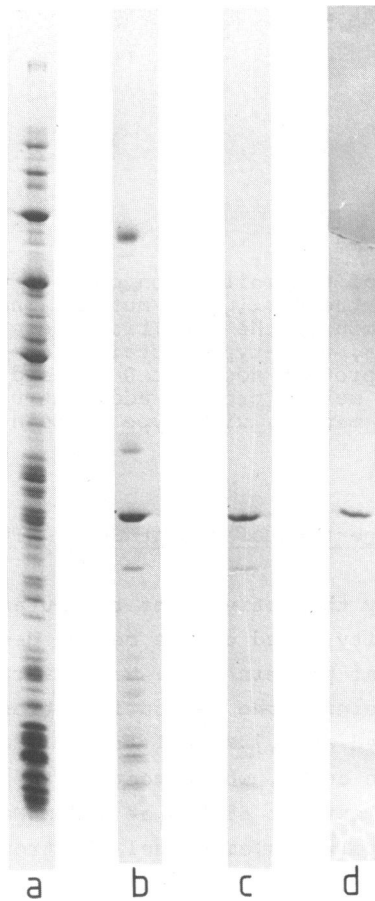


Figure 4. SDS-polyacrylamide gel electrophoresis of the purification steps of the Ser₂₃₂→Thr mutant of the ϕ 29 terminal protein. Proteins at various purification steps were subjected to SDS-gel electrophoresis in a 10-20% acrylamide gradient. Lanes: a, extract (50 μ g); b, protein eluted at 0.7 M NaCl from a DEAE-cellulose column; nucleic acid was removed by polyethyleneimine precipitation and protein was concentrated by ammonium sulfate precipitation (7.5 μ g); c, protein eluted from a phosphocellulose column with 1 M NaCl (4.3 μ g); d, protein eluted from the second DEAE-cellulose column at 0.3 M NaCl (1.3 μ g).

tiation complex was observed in extracts from cells containing plasmid p3S₂₃₂, but not in those with plasmid p3T₂₃₂ (not shown), suggesting that the mutation Ser₂₃₂→Thr inactivates the terminal protein for the priming reaction.

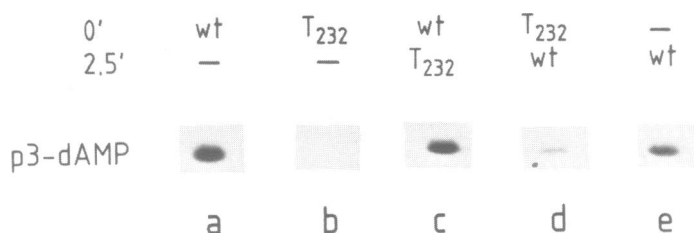


Figure 5. Formation of the initiation complex with the wild-type terminal protein and the Ser₂₃₂→Thr mutant. The reaction mixture was incubated for 5 min in the conditions described in Materials and Methods. Lanes: a, wild-type protein alone; b, mutant protein alone; c, wild-type protein added at 0 min and mutant protein added at 2.5 min; d, mutant protein added at 0 min and wild-type protein added at 2.5 min; e, wild-type protein alone added at 2.5 min.

Purification and properties of mutant Ser₂₃₂→Thr of the Ø29 terminal protein

To further study the activity of the mutant protein, it was purified to homogeneity based on the method developed to purify the wild-type terminal protein (30). No differences were observed between the two proteins along the purification procedure; mutant protein p3 also eluted at 0.7 M NaCl from a DEAE-cellulose column together with nucleic acids, while most of the protein contaminants present in the extract eluted at 0.3 M NaCl. Fig. 4 shows the analysis by SDS-polyacrylamide gel electrophoresis of the purification steps of the mutant protein p3.

Purified protein p3 containing the mutation Ser₂₃₂→Thr did not show any activity in the formation of the initiation complex (Fig. 5, lane b), even in conditions in which less than 0.1% of the wild-type activity could have been detected. On the other hand, the amount of initiation complex formed in 5 min with the wild-type protein alone (Fig. 5, lane a) was higher than that obtained when the mutant protein was added after 2.5 min (Fig. 5, lane c). In addition, the amount of p3-dAMP complex formed in 2.5 min with the wild-type protein (Fig. 5, lane e) was much higher than that obtained when the mutant protein was previously added (Fig. 5, lane d). When both proteins were added at the same time, the initiation complex formed was about half of that formed by the wild-type protein alone (not shown). These results suggest that the mutant terminal protein competes with the wild-type

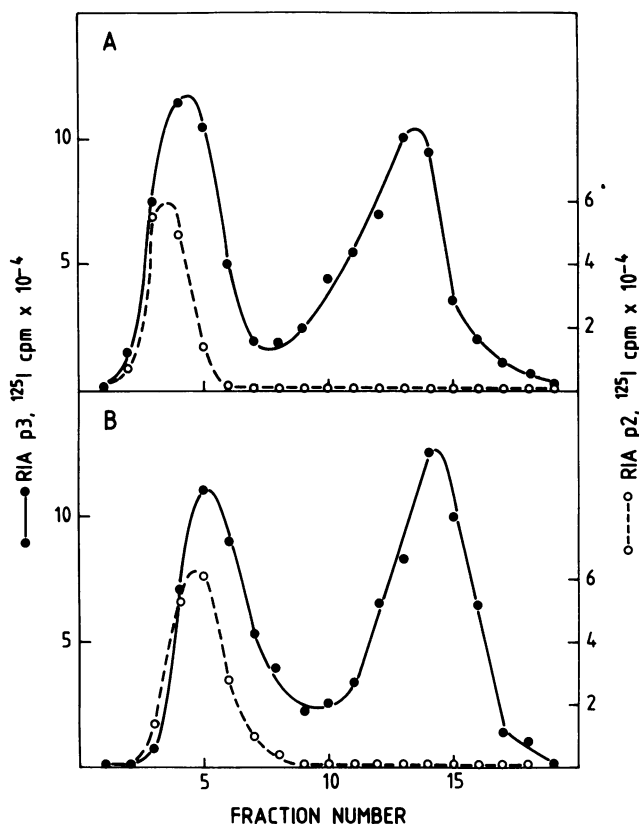


Figure 6. Interaction of the wild-type terminal protein and the Ser₂₃₂→Thr mutant with the ϕ 29 DNA polymerase. Wild-type terminal protein (0.6 μ g) or Ser₂₃₂→Thr mutant (0.6 μ g) was incubated with 0.6 μ g of ϕ 29 DNA polymerase for 1 h at 0°C in a final volume of 0.2 ml in a buffer containing 50 mM Tris-HCl, pH 7.5, 44 mM NaCl and 20 mM (NH₄)₂SO₄. The samples were layered on top of a 5 ml linear 15–30% glycerol gradient in the above buffer and centrifuged for 28 h at 290,000 \times g at 4°C. Fractions were collected from the bottom and terminal proteins and DNA polymerase were detected by radioimmunoassay (RIA) as described (30). A, wild-type terminal protein. B, mutant terminal protein. Sedimentation was from right to left.

protein in the interaction with the DNA polymerase and/or with the DNA. The interaction between the terminal protein and the ϕ 29 DNA polymerase, in the presence of NH₄⁺ ions, can be detected by glycerol gradient centrifugation (11). The formation of such a complex with the mutant terminal protein was studied to determine

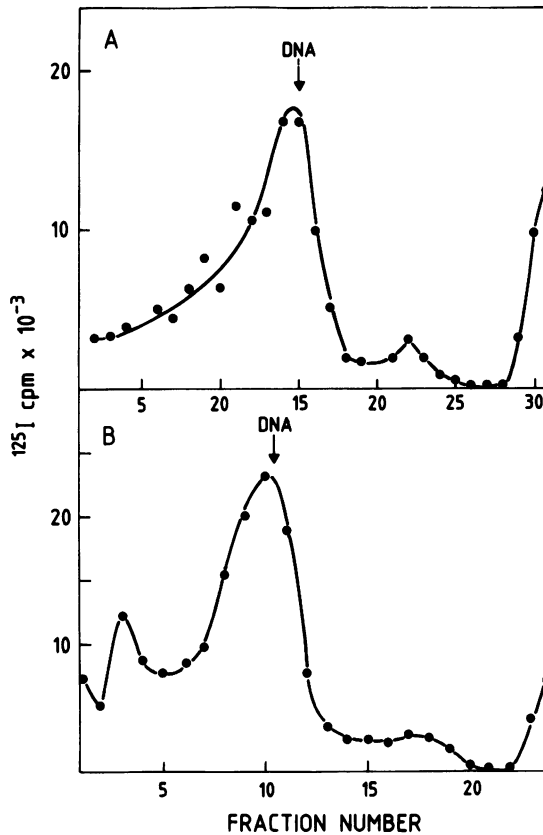


Figure 7. Interaction of the wild-type terminal protein and the $\text{Ser}_{232}\text{Thr}$ mutant with DNA. Wild-type terminal protein ($0.6 \mu\text{g}$) or $\text{Ser}_{232}\text{Thr}$ mutant ($0.6 \mu\text{g}$) was incubated with $20 \mu\text{g}$ of $\phi 29$ DNA for 1 h at 0°C in a final volume of 0.2 ml in the buffer described in the legend to Fig. 6 containing 1 mM EDTA, and subjected to glycerol gradient centrifugation for 4 h at $260,000 \times g$ as described in the legend to Fig. 6. Fractions were collected from the bottom. The position of DNA, indicated by an arrow, was determined by agarose gel electrophoresis and ethidium bromide staining. The terminal protein was detected by radioimmunoassay as described (30). A, wild-type terminal protein. B, $\text{Ser}_{232}\text{Thr}$ mutant. Sedimentation was from right to left.

whether the $\text{Ser}_{232}\text{Thr}$ mutation affects the site of interaction with the DNA polymerase. Fig. 6 shows that the mutant terminal protein interacts with the $\phi 29$ DNA polymerase in the same extent as the wild-type protein.

The phage $\phi 29$ terminal protein or its complex with the DNA

polymerase must recognize the terminal sequences of ϕ 29 DNA since the initiation complex can be formed in protein-free templates containing at least the 12 base pairs of the ϕ 29 DNA ends (14). However, no specific interaction of the terminal protein, by itself or in the presence of the DNA polymerase, with ϕ 29 DNA terminal fragments has been obtained so far. Instead, unspecific interaction of protein p3 with double-stranded DNA has been obtained (32). Figure 7 shows that the Ser₂₃₂→Thr mutant of protein p3 cosediments with DNA in a glycerol gradient in a way similar to that of the wild-type protein.

The results presented here indicate that the priming residue of the ϕ 29 terminal protein is highly specific and the mutation Ser₂₃₂→Thr seems to affect the ability of the ϕ 29 DNA polymerase to catalyze the formation of the covalent complex with dAMP. It would be interesting to find ϕ 29 DNA polymerase mutants able to form the initiation complex with the mutant terminal protein.

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