
Characterization of a 3' → 5' exonuclease activity in the phage ϕ 29-encoded DNA polymerase

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ABSTRACT

Purified protein p2 of phage ϕ 29, characterized as a specific DNA polymerase involved in the initiation and elongation of ϕ 29 DNA replication, contains a 3' → 5' exonuclease active on single-stranded DNA, but not on double-stranded DNA. No 5' → 3' exonuclease activity was found. The 3' → 5' exonuclease activity was shown to be associated with the DNA polymerase since 1) the two activities were heat-inactivated with identical kinetics and 2) both activities, present in purified protein p2, cosedimented in a glycerol gradient.

INTRODUCTION

The Bacillus subtilis bacteriophage ϕ 29 has a linear double-stranded DNA with a protein of 31,000 daltons covalently linked at the 5' ends (1-4). The terminal protein p3 is the product of the viral gene 3, and is linked to the DNA by a phosphoester bond between the OH group of a serine residue and 5'-dAMP (5). ϕ 29 replication is initiated at either DNA end (6,7) by a protein-priming mechanism in which a free molecule of the terminal protein p3 reacts with dATP to form a protein p3-dAMP covalent complex that provides the 3'OH group needed for elongation (8,9). The initiation reaction requires, in addition to the gene 3 product and ϕ 29 DNA-protein p3 as template, the product of the viral gene 2 (10-12), which was shown to be a DNA polymerase (13-15). By in situ gel analysis the DNA polymerase activity was shown to be associated with the protein p2 band of molecular weight 68,000 daltons (14). When highly purified proteins p2 (DNA polymerase) and p3 (terminal protein) were used in an in vitro initiation system, formation of the p3-dAMP initiation complex was greatly stimulated by extracts from uninfected B. subtilis or E. coli cells (14). In addition to its role in the initiation reaction, the ϕ 29

DNA polymerase was able to elongate at least up to nucleotides 9 and 12 from the left and right DNA ends, respectively (14,15).

In this paper we report that purified protein p2 has a 3' → 5' exonuclease activity on single-stranded but not on double-stranded DNA. Both the 3' → 5' exonuclease activity and the DNA polymerase activity are shown to be associated since they are heat-inactivated with identical kinetics and the two activities present in purified protein p2 cosediment in a glycerol gradient.

MATERIALS AND METHODS

Purification of protein p2

Protein p2 was purified from E. coli cells harbouring a gene 2-containing recombinant plasmid as described (12,14). After elution from a phosphocellulose column, the last purification step, the protein was about 86% pure giving essentially a single band of molecular weight 68,000 daltons by SDS-polyacrylamide gel electrophoresis (14). When indicated, the phosphocellulose fraction of protein p2 (2.4 µg) was further subjected to centrifugation for 19 h at 380,000 x g in a 15-30% (v/v) glycerol gradient in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl.

Assay for formation of the p3-dAMP initiation complex

The incubation mixture for the initiation reaction contained, in 0.05 ml, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1 mM spermidine, 0.25 µM {α-³²P}dATP (5 µCi; 410 Ci/mmol), 1 µg of Ø29 DNA-protein p3, 20 ng of purified protein p3 (16) obtained from I. Prieto, 2 µg of a partially purified fraction from E. coli cells as a source of host factor (14) and fractions from a glycerol gradient containing protein p2 purified as described (14). After incubation for 20 min at 30°C the samples were processed as described by Peñalva and Salas (8).

Replication assay with Ø29 DNA-protein p3 as template

The replication assay consists in the elongation of the protein p3-dAMP initiation complex formed when purified proteins p2 (DNA polymerase) and p3 (terminal protein) are incubated in the presence of host factor and Ø29 DNA-protein p3 as a template (14). The incubation mixture was as described for the initiation reaction except that it contained 20 µM {α-³²P}dATP (2 µCi)

and 20 μ M dGTP, dCTP and dTTP as well as 1 mM ATP, 5% (v/v) glycerol and bovine serum albumin (0.1 mg/ml). After 20 min at 30°C, the reaction was stopped by addition of 10 mM EDTA/0.1% SDS and heating for 10 min at 68°C and the samples were filtered through Sephadex G50 spun-columns as described (17). The excluded fraction was counted by Cerenkov radiation.

Exonuclease assay

The incubation mixture contained, in 0.05 ml, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM ATP, 5 mM 2-mercaptoethanol, the amount of purified protein p2 indicated in each case and either {3'-³²P}-DNA or {5'-³²P}-DNA (5000 cpm), native or heat-denatured. After incubation for 20 min at 37°C the ethanol-soluble material was counted by Cerenkov radiation. When indicated, the ethanol precipitate was dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and subjected to electrophoresis in a 8% polyacrylamide gel as described (18). After electrophoresis the gel was dried and autoradiographed with intensifying screens at -70°C. The {3'-³²P}-DNA used was prepared by labelling restriction fragments with either terminal deoxynucleotidyl transferase and { α -³²P} dideoxy ATP (gift from C. Escarmís) or with *E. coli* DNA polymerase I (Klenow fragment) and { α -³²P}dATP (gift from R. Blasco). The {5'-³²P}-DNA was obtained by labelling with polynucleotide kinase and { γ -³²P} ATP (gift from C. Escarmís).

RESULTS

3' \rightarrow 5' exonuclease activity of ϕ 29 DNA polymerase

DNA labelled at the 3' end or at the 5' end with ³²P was incubated, either in native or denatured form, with purified protein p2 and the ethanol-soluble radioactivity determined. As shown in Figure 1A, incubation of protein p2 with denatured but not with native {3'-³²P}-DNA produced the release of ethanol-soluble radioactivity. No radioactivity was hydrolyzed from denatured or native {5'-³²P}-DNA (Fig. 1B). These results suggested that protein p2 has 3' \rightarrow 5' nuclease activity. This was in agreement with the fact that the insoluble material remaining after treatment of denatured {3'-³²P}-DNA with protein p2 had the same size as the control samples, as determined by polyacrylamide gel electrophoresis, but the amount of radioactivity decreased with

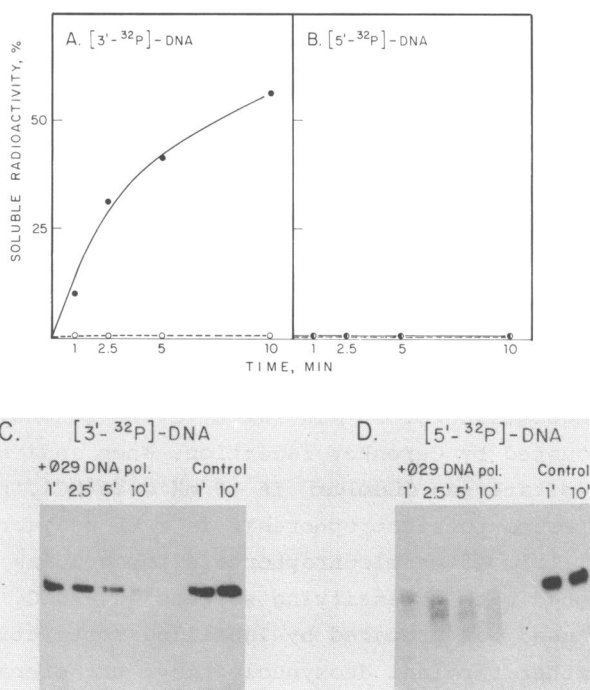


Fig.1. 3' → 5' exonuclease activity of the phi29 DNA polymerase. Purified protein p2 (10 ng) was incubated for the indicated times with native (o--o) or heat-denatured (●—●) DNA, labelled at the 3' end with {alpha-³²P}dideoxy ATP (5000 cpm, 26 ng of DNA) (A) or at the 5' end with {gamma-³²P}ATP (5000 cpm, 13 ng of DNA) (B) as described in Materials and Methods, and the ethanol-soluble radioactivity from the incubations with denatured DNA shown in A and B, respectively, was subjected to polyacrylamide gel electrophoresis as described in Materials and Methods. Controls of samples incubated in the absence of protein p2 were also subjected to electrophoresis. The {3'-³²P}- and {5'-³²P}-DNA fragments were 600 and 900 nucleotides long, respectively.

the incubation time (Fig. 1C). Moreover, the size of the insoluble material which remained after treatment of denatured {5'-³²P}-DNA with protein p2 decreased with the incubation time although the amount of radioactivity remained constant (Fig. 1D). The above results indicate the existence of a 3' → 5' nuclease activity on single-stranded DNA in purified protein p2.

Evidence that the 3' → 5' nuclease is an exonucleolytic activity was obtained from the analysis of the soluble radioactivity released from DNA labelled at the 3' end with {alpha-³²P}dATP

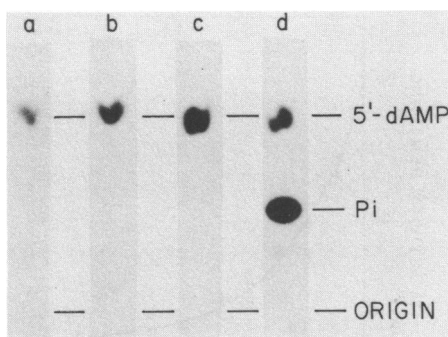


Fig. 2. Characterization of the nucleotide released after treatment of $\{3'-^{32}\text{P}\}$ -DNA with protein p2. $\{3'-^{32}\text{P}\}$ -DNA prepared by labelling with $\{\alpha-^{32}\text{P}\}$ dATP (4000 cpm, 0.7 ng of DNA) was heat-denatured and incubated with 20 ng of purified protein p2 for 1, 2.5 and 10 min as described in Materials and Methods and the ethanol-soluble radioactivity determined. A sample of the latter fraction was applied to a polyethyleneimine-cellulose plate; the chromatogram was developed with 0.15 M lithium formate, pH 3.0. In all cases 5'-dAMP was run as an internal marker. a) 1 min; b) 2.5 min; c) 10 min; d) as b plus ^{32}P i as marker.

at several times of incubation with protein p2. As shown in Fig. 2, the hydrolyzed radioactivity was characterized as dAMP by thin layer chromatography. No oligonucleotides were released, even at short incubation times.

Association of the replication and 3' \rightarrow 5' exonuclease activities of the ϕ 29 DNA polymerase

Evidence that the DNA polymerase and 3' \rightarrow 5' exonuclease activities of purified protein p2 are associated was obtained from the fact that the two activities were inactivated at 42°C with identical kinetics, as shown in Figure 3.

A further evidence that the two activities are associated was obtained when purified protein p2 was subjected to glycerol gradient centrifugation. As shown in Fig. 4A the DNA polymerase activity associated with protein p2 cosedimented with the 3' \rightarrow 5' exonuclease activity at a position corresponding to a molecular weight of 68,000 daltons, previously shown by *in situ* gel analysis to be the size of the ϕ 29 DNA polymerase encoded by gene 2 (14). In addition, the initiation activity assayed as the formation of the p3-dAMP complex (14) was also present in the same protein p2 peak (Fig. 4B).

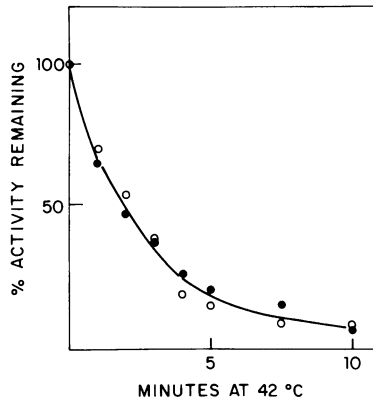


Fig. 3. Co-inactivation of replication and 3' \rightarrow 5' exonuclease activities of the ϕ 29 DNA polymerase. Purified protein p2 was incubated for the indicated times at 42°C in a buffer containing 25 mM Tris-HCl, pH 7, 0.2 M NaCl, 50% glycerol and bovine serum albumin (1 mg/ml). Samples (20 ng of protein p2) were removed at the indicated times and assayed for ϕ 29 DNA-protein p3 replication and single-strand 3' \rightarrow 5' exonuclease activities as described in Materials and Methods using {3'- 32 P}DNA prepared by labelling with { α - 32 P}dATP (3000 cpm, 7.2 ng of DNA). ●—●, replication activity; o---o, exonuclease activity.

Requirements for the 3' \rightarrow 5' exonuclease activity

As shown in Table 1, when the Mg^{++} is omitted from the reaction mixture the exonuclease activity is reduced to less than 1%. In the absence of mercaptoethanol the activity is slightly reduced and ATP has no effect. Addition of other components used in the replication assay (spermidine, glycerol and bovine serum albumin) had also no effect. The salt affects the exonuclease activity; addition of 0.35 M KCl results in 85% inhibition. Deoxyribo or ribonucleoside-5'-triphosphates or monophosphates essentially did not change the exonuclease activity.

To get an idea on the magnitude of the exonuclease activity of the ϕ 29 DNA polymerase, 25 ng of denatured ϕ 29 DNA, uniformly labelled, was used as a substrate. A value of 23 fmol of nucleotide hydrolyzed/min/ng of protein p2 was obtained. The polymerizing activity of protein p2 assayed with poly(dA)-(dT)₁₂₋₁₈ as template-primer (14) was 30 fmol of dTMP incorporated/min/ng of protein p2. When ϕ 29 DNA-protein p3 was used as template in a replication assay as described in Materials and Methods the ac-

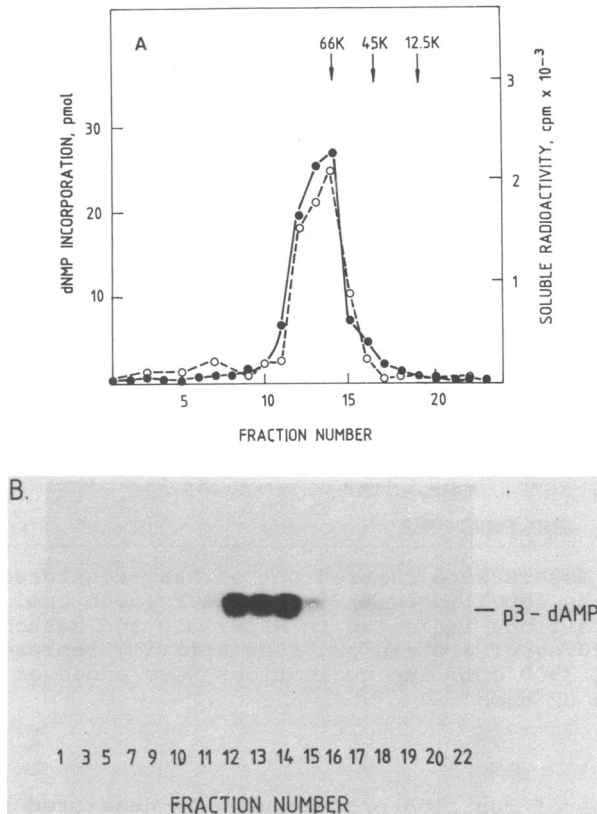


Fig.4. Glycerol gradient centrifugation of the $\phi 29$ DNA polymerase. Purified protein p2 (2.4 μg) was subjected to centrifugation in a 15-30% (v/v) glycerol gradient as described in Materials and Methods. 50 μg of bovine serum albumin, ovalbumin and cytochrome C were added as molecular weight markers. Fractions were taken and a sample from each fraction was used to analyze for DNA polymerase activity with $\phi 29$ DNA-protein p3 as template ($\bullet\text{---}\bullet$) and 3' \rightarrow 5' exonuclease activity using $\{3'\text{-}^{32}\text{P}\}$ DNA prepared by labelling with $\{\alpha\text{-}^{32}\text{P}\}$ dATP (4000 cpm, 0.7 ng of DNA) ($\text{o}\text{---}\text{o}$) (A) and for the formation of the p3-dAMP initiation complex (B) as described in Materials and Methods. The arrows indicate the position of the protein markers.

tivity of the $\phi 29$ DNA polymerase was 285 fmol of nucleotide incorporated/ $\mu\text{min}/\text{ng}$ of protein p2. The last value could be higher if the formation of the p3-dAMP initiation complex is rate-limiting in the replication assay.

Table 1. Requirements of the 3' → 5' exonuclease of the ϕ 29 DNA polymerase

System	Activity, %
Complete	100
- Mg ⁺⁺	0.7
- 2-mercaptoethanol	72
- ATP	114
+ 1 mM spermidine + 5% (v/v) glycerol	
+ bovine serum albumin (0.1 mg/ml)	100
+ 0.15 M KCl	65
+ 0.25 M KCl	35
+ 0.35 M KCl	15
+ dATP, dGTP, dCTP, dTTP	83
+ ATP, GTP, CTP, UTP	102
+ dAMP, dGMP, dCMP, dTMP	103
+ AMP, GMP, CMP, UMP	103

The nuclease assays were carried out on heat-denatured {3'-³²P}-DNA prepared by labelling with { α -³²P}dATP (5000 cpm, 12 ng of DNA) under the conditions indicated in Materials and Methods in the presence of 20 ng of protein p2. 100% activity represents the hydrolysis of 3500 cpm. The nucleotides were added at a concentration of 30 μ M each.

DISCUSSION

Incubation of purified protein p2 with denatured but not with native {3'-³²P}-DNA released ethanol-soluble radioactivity. No radioactivity was hydrolyzed from denatured or native {5'-³²P}-DNA. These results suggest that protein p2 has 3' → 5' nuclease activity. Evidence that the nuclease activity of protein p2 was exonucleolytic was obtained from the fact that free dAMP, and not oligonucleotides, was released at different incubation times. DNA labelled at the 3' end either with deoxy ATP or di-deoxy ATP could be used as substrate for the 3' → 5' nuclease activity indicating that the enzyme is not very specific for the deoxyribo configuration, unlike the 3' → 5' exonuclease activity of *E. coli* DNA polymerase I (19).

Further evidence that the 3' → 5' exonuclease activity was indeed associated with the DNA polymerase encoded by gene 2 of phage ϕ 29 was obtained from the fact that both activities were heat-inactivated with identical kinetics and the two activities,

present in purified protein p2, cosedimented in a glycerol gradient. As has been shown previously (14,15), the ϕ 29 DNA polymerase is required for the initiation of ϕ 29 DNA replication, most likely catalyzing the formation of the covalent linkage between the terminal protein p3 and 5'-dAMP. In agreement with that, the ability to catalyze the formation of the p3-dAMP complex (initiation activity), also cosedimented in the glycerol gradient with the replication activity and with the 3' \rightarrow 5' exonuclease activity of protein p2.

As other 3' \rightarrow 5' exonucleases associated to DNA polymerases, the activity present in the ϕ 29-encoded DNA polymerase requires Mg^{++} and it is inhibited by high salt. Deoxynucleoside-5'-triphosphates essentially did not affect the 3' \rightarrow 5' exonuclease activity of the ϕ 29 DNA polymerase, as has been also reported for the 3' \rightarrow 5' exonuclease activity on single-stranded DNA of E. coli DNA polymerase I (19) and in contrast with the inhibition by deoxyribonucleoside-5'-triphosphates of the 3' \rightarrow 5' exonuclease activity on single-stranded DNA of the T7 DNA polymerase (20,21). Ribonucleoside-5'-triphosphates or nucleoside-5'-monophosphates, in the deoxy or ribo configuration, essentially had no effect on the 3' \rightarrow 5' exonuclease of ϕ 29 DNA polymerase.

The 3' \rightarrow 5' exonuclease of the ϕ 29 DNA polymerase is very specific for single-stranded DNA as is the case with the E. coli DNA polymerases I and III (19) and with the T4 DNA polymerase (22). Like the T4, T5 and T7 DNA polymerases (23), the ϕ 29 DNA polymerase does not have 5' \rightarrow 3' nuclease activity. It remains to be determined whether ϕ 29 induces a 5' \rightarrow 3' exonuclease activity different from the DNA polymerase, as happens after T5 and T7 infection (23). However, since ϕ 29 DNA replication is initiated by protein-priming and discontinuous synthesis initiated by RNA priming does not seem to be needed, it is likely that a specific 5' \rightarrow 3' nuclease activity to remove the RNA primers is not necessary.

As has been suggested by Brutlag and Kornberg (24), the 3' \rightarrow 5' exonuclease activity on single-stranded DNA of DNA polymerases provides a proofreading function to maintain the fidelity of template copying at a high level. Evidence that will be

presented elsewhere indicates that the ϕ 29 DNA polymerase is able to elongate the p3-dAMP initiation complex, not only up to oligonucleotides 9 and 12 bases long, but also to full length ϕ 29 DNA. Therefore it seems that, at least in vitro, the ϕ 29-encoded DNA polymerase is the only enzyme involved in catalyzing the polymerization of ϕ 29 DNA. It is therefore interesting to point out that such a novel DNA polymerase that is able to function at the initiation and elongation steps in replication, has also the 3' \rightarrow 5' exonuclease activity on single-stranded DNA that acts most likely as a proofreading mechanism.

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