# Characterization of a DNA binding protein of bacteriophage PRD1 involved in DNA replication

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## ABSTRACT

*Escherichia coli* **phage PRD1 protein P12, involved in PRD1 DNA replication** *in vivo*, **has been highly purified from** *E. coli* **cells harboring a gene XII-containing plasmid. Protein P12 binds to single-stranded DNA as shown by gel retardation assays and nuclease protection experiments. Binding of protein P12 to single-stranded DNA increases about 14% the contour length of the DNA as revealed by electron microscopy. Binding to single-stranded DNA seems to be cooperative, and it is not sequence specific. Protein P12 also binds to double-stranded DNA although with an affinity 10 times lower than to single-stranded DNA. Using the** *in vitro* **phage** $\phi$29 **DNA replication system, it is shown that protein P12 stimulates the overall** $\phi$29 **DNA replication.**

## INTRODUCTION

PRD1 is a lipid-containing bacteriophage infecting a variety of Gram-negative bacteria harboring an appropriate plasmid. Among the hosts are *Escherichia coli* and *Salmonella typhimurium* (for review see 1). The genome of the phage is a linear 14.9 kb double-stranded DNA molecule, which has inverted terminal repeats of 110 bp (2,3), and a terminal protein (protein P8) covalently linked to the 5'ends (4,5,6,7,8). The genome is replicated by a protein-primed mechanism (reviewed in 9), in which the 5' terminal nucleotide (dGMP) is covalently attached to a tyrosine residue of the terminal protein (5), probably catalyzed by the viral polymerase (protein P1). Both the DNA polymerase and the terminal protein together with proteins P12, P19 and P15 are viral early gene products. Protein P12 has been shown to be required for PRD1 DNA replication and shut-off of early protein synthesis (10). Genetic analysis indicated that P15 is a lytic enzyme (10), while the function of protein P19 is still unknown.

The best understood protein primed replication systems are those of adenovirus (reviewed in 11) and *Bacillus subtilis* phage $\phi$29 (9). In addition to DNA polymerase and terminal protein these viruses code for additional factors needed for replication, including single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) binding proteins. Adenoviruses code for a

multifunctional protein, DBP, which is involved in replication, transcription control, assembly and transformation. It binds to ssDNA and, with lower efficiency, also to dsDNA (for reviews see 11,12). In adenovirus replication DBP cooperates with the DNA polymerase in the displacement of the non-template strand, and binds to the displaced ssDNA (13,14). DBP also binds to dsDNA and enhances binding of NFI to the adenovirus DNA terminal sequences, thereby stimulating the initiation step of DNA replication (15,16). Phage $\phi$29 codes for a ssDNA binding protein (SSB), protein p5, that stimulates *in vitro* $\phi$29 DNA replication (17) mainly by increasing the ability of $\phi$29 DNA polymerase to reinitiate replication, overcoming the inhibition due to the increasing amount of ssDNA produced in the reaction (18). A direct effect of p5 on the initiation step of $\phi$29 DNA replication has not been observed (17). Phage $\phi$29 also codes for a dsDNA binding protein, protein p6, which specifically binds to the $\phi$29 DNA terminal sequences, stimulating the initiation of $\phi$29 DNA replication (19).

In this study we have purified protein P12 of phage PRD1 and we have shown that it binds to ssDNA and, with lower efficiency, also to dsDNA. We have also shown that protein P12 stimulates *in vitro* $\phi$29 DNA replication.

## MATERIALS AND METHODS

### Bacterial strains, phages and plasmids

Phage PRD1 (20) was grown on *S. typhimurium* DS88 (21) harboring the receptor coding plasmid pLM2 (22), concentrated, and purified as described previously (23). PRD1 nonsense mutant in gene XII (*sus*156) was grown on suppressor strain *S.typhimurium* DB7156 (10). Phage DNA was extracted after pronase treatment as previously described (24). The standard DNA manipulations were done according to (25).

Plasmid pTPH37 (26) that contains genes XII and XIX cloned into vector pPLH101 (27) under the control of lambda $P_L$ promoter, was deleted by exonuclease III treatment (28) to remove gene XIX. The clones obtained were sequenced using the Sanger dideoxy method modified for dsDNA (29) and for T7 DNA polymerase (30). Plasmid pTPH12 containing nucleotides 228−787 from the right end of the genome (for nucleotide numbering see 26) was used for expression of gene

XII. Plasmid pTPH12 was amplified on either *E. coli* DH5α harboring the plasmid pcI857 (31) or *E. coli* PK466 (8).

The terminal 315 bp fragment of the left end of PRD1 genome was cloned into *Hinc2* restriction site of plasmid pUC18 by H. Savilahti. For the DNA binding and nuclease protection assays the fragment was isolated using the adjacent restriction sites, *Xba1* and *Pst1*, in the polylinker site.

## Complementation test

PRD1 *sus*156 production in *E. coli* PK466 cells harboring either plasmids pLM2 and pTPH12, or as a control, plasmids pLM2 and pPLH101, was quantitated by a one step growth-like procedure as follows: Both bacterial strains were heat-induced (42°C) at a cell density of 5×10⁸ cfu/ml for 25 min (see below), and then infected at a multiplicity of 0.1. After 5 min, the remaining unadsorbed phages were inactivated by diluting the infection mixture 1:10 in growth media containing 1% of anti-PRD1 polyclonal rabbit serum. After a 5 min incubation with the antibody, the mixture was further diluted 1:1000 in prewarmed growth media and the culture was incubated at 42°C with vigorous shaking. Samples were taken every 10 min and phage titers were determined on the suppressor strain *S. typhimurium* DB7156.

## Overproduction and purification of protein P12

*E. coli* DH5α(pTPH12, pcI857) was grown at 27°C to cell density of 5×10⁸ cfu/ml, then the temperature was raised to 42°C to induce the lambda P_L promoter and after 3 h at 42°C cells were harvested. Cellular proteins were analyzed before and after induction by SDS-PAGE (as described in 32). Control cells harboring the plasmid vector without phage DNA insert were grown and analyzed in the same way. For protein purification, cells from a 2 liter culture were harvested, resuspended into 15ml of 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂ and 5% glycerol, and disrupted in French press. The cell debris was removed by centrifugation (15 min, 20 000 g) and then the supernatant was further centrifuged (4 h, 120 000 g). The soluble fraction was applied to a 6 ml heparin-agarose column. After washing the column with the same buffer, adsorbed proteins were eluted by a linear NaCl gradient from 50 mM upto 1 M. Samples containing P12 were pooled and dialyzed overnight against 50 mM Tris-HCl, pH 7.5 and 50 mM NaCl. The P12 preparation was then passed through a 5 ml DEAE-agarose column. The flow-through containing P12 was applied to a 2 ml hydroxyapatite column equilibrated with the same buffer. Protein P12 was eluted with 100 mM K-phosphate buffer, pH 7.5 and concentrated to about 5 mg/ml by dialysis against solid PEG 20 000. Glycerol was added up to 30% and samples were stored in aliquots at −70°C. Approximately 10−12 mg of protein could be obtained from a 2 liter culture. The molecular weight of the native protein P12 was determined by centrifugation of purified protein P12 (rotor SW41, 35 000 rpm, 6°C, 48 h) in a 5%−20% sucrose gradient in 50 mM Tris-HCl, pH 7.5 and 50 mM NaCl. Rabbit muscle aldolase (158 kDa), bovine serum albumin (67 kDa) and horse heart cytochrome c (12 kDa) were used as molecular weight markers.

## Gel retardation assays

Gel retardation assays were carried out essentially as previously described (33). Protein P12, at the indicated amounts, was incubated 30 min on ice with end labelled 315 bp DNA fragment (∼5 ng, ∼500cpm) in 20 μl of 50 mM Tris-HCl pH 7.5,

50 mM NaCl, 20 mM (NH₄)₂SO₄, 10 mM MgCl₂, 1 mM DTT and 2μg BSA. When binding to ssDNA was assayed, the dsDNA fragment was denatured by heating for 3 min at 95°C before incubation with the protein. The protein-DNA complexes were resolved in 4% polyacrylamide gels (acrylamide: bis-acrylamide 80:1). After electrophoresis in TBE buffer (25) the gels were dried and autoradiographed.

## Protection of ssDNA against nuclease P1

Heat-denatured nick-translated DNA fragment (∼10 ng) from the left terminus of PRD1 genome was incubated with the indicated amounts of protein P12 in 30 mM Tris-acetate, pH 7.5, 10 mM MgCl₂, and 0.1 mM ZnCl₂ as described in (17). Protein-DNA mixture (total volume 25μl) was incubated 30 min on ice before treatment with nuclease P1 (4μg) for 5 min at 30°C. The reaction was stopped by adding 25μl of tRNA (0.4 mg/ml) and 50μl of 14% ice cold TCA. Samples were kept on ice for 45 min and centrifuged 15 min in an Eppendorf centrifuge. Pellets were washed once and the radioactivity in supernatants and pellets was determined.

## Electron microscopy

Protein P12 (6μg) was incubated with M13mp8 ssDNA (mass ratio 40:1) in the buffer used for in vitro φ29 p3-DNA replication (described below). After 20 min at 30°C, free protein was removed by gel filtration through a Sepharose CL 4B column equilibrated with 7 mM magnesium acetate. Fractions were collected in tubes containing glutaraldehyde to give a final
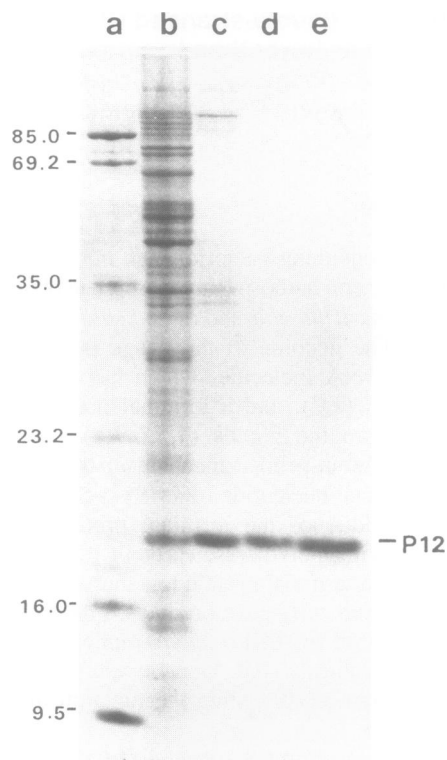


**Figure 1.** Purification of protein P12. Samples at different purification steps analyzed by SDS-PAGE: lane a) proteins of phage φ6 used as molecular weight markers (1), b) disrupted cells after centrifugations, c) P12-containing fractions from the heparin-agarose column, d) flow-through from the DEAE-agarose column, e) P12 eluted from the hydroxyapatite column. The position of P12 is indicated on the right.

concentration of 0.1%. After 1 hour at room temperature, samples were prepared for electron microscopy according to the BAC spreading technique (34). Single stranded M13 DNA was added just before spreading and was used as an internal control for contour length measurements. Samples were picked up with standard carbon coated grids pretreated with uranyl acetate and rotary shadowed with platinum. Micrographs were taken in a Philips 420 electron microscope at 100 kV and at a magnification of 18500×. Contour length was determined in a HP Digitizer.

### Replication assay using φ29 DNA

Replication assays with φ29 DNA-terminal protein as template using purified φ29 DNA polymerase (p2) and φ29 terminal protein (p3) were carried out as described in (17). The incubation mixture (total volume 20μl) contained 50 mM Tris-acetate, pH 7.5, 10 mM Mg-acetate, 1 mM dithiothreitol, 10 mM $(NH_4)_2SO_4$, 20 mM NaCl, 25μg bovine serum albumin, φ29 DNA-terminal protein (220 ng), 40μM each dCTP, dGTP, dTTP and [α-$^{32}$P]dATP (2μ Ci) and purified proteins p2 (24 ng) and p3 (15 ng). Incubation was carried out at 30°C for the indicated times in the absence or presence of the indicated amounts of PRD1 protein P12. Reactions were stopped with 10 mM EDTA and 0.1% SDS, filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, and the Cerenkov radiation of the excluded volume was measured. When the rate of elongation was to be determined, the labelled DNA from the excluded volume was subjected to gel electrophoresis in 0.7% alkaline agarose gels.

Initiation of the replication was assayed essentially as described above except that [α-$^{32}$P]dATP (2μ Ci) was the only dNTP present. After 10 min at 30°C the reaction was stopped, and samples were treated with micrococcal nuclease and subjected to SDS-polyacrylamide gel electrophoresis.

## RESULTS AND DISCUSSION

### Expression and purification of protein P12

The ability of plasmid pTPH12, containing gene XII under the control of lambda $P_L$ promoter, to produce biologically active

protein P12 was analyzed by complementation of a nonsense mutation in gene XII. Due to the leakiness of the *sus*156 mutant, standard complementation could not be carried out, but instead an one step growth-like experiment was done. *E. coli* PK466 non-suppressor cells harboring plasmids pLM2 and pTPH12, or control cells harboring plasmids pLM2 and pPLH101, were infected with phage PRD1 *sus*156, and phage titers of the infected cultures were determined on a suppressor host at different times after infection. At 90 min post-infection, the phage production was 7−10 times higher in cells harboring pTPH12 than in control cells. Therefore, protein P12 produced from the plasmid during the heat induction is able to complement the *sus*156 PRD1 mutation.

Purification of protein P12 was carried out as described in Materials and Methods and is summarised in Fig. 1. Most of the contaminant proteins are removed during the chromatography on heparin-agarose. The few contaminants still left were removed by the DEAE-agarose and hydroxyapatite chromatography. The P12 obtained was at least 95% pure. Degradation of P12 was observed depending on the particular *E. coli* strain used for the expression. *E. coli* PK466 produced an approximately 13 kDa fragment in addition to the native protein P12. The degradation was markedly deminished when *E. coli* DH5α(pCI857) was used as a host. The degradation product was identified as a P12 derivative by gel electrophoresis analysis of samples incubated at room temperature in the absence or presence of the proteinase inhibitor PMSF. This degradation product can be separated from P12 during the heparin-agarose step, since it elutes at lower NaCl concentration than P12.

By sucrose gradient centrifugation, native protein P12 had an apparent molecular weight about 12 kDa. The molecular weight determined from the DNA sequence is 16,7 kDa (26). The sedimentation behaviour of P12 is consistent with monomeric state.

### DNA binding properties of protein P12

To test the ability of P12 to bind to DNA, gel retardation assays were carried out with different amounts of P12 and either ssDNA or dsDNA (Fig 2.). Protein P12 formed a well defined complex with ssDNA that moved at the same position as P12 alone, as determined by Coomassie-blue staining of the retarding gel. The minimal amount of protein P12 needed to change the mobility of the ssDNA fragment was around 150 ng; a relatively small increase in the amount of the protein was needed to retard all
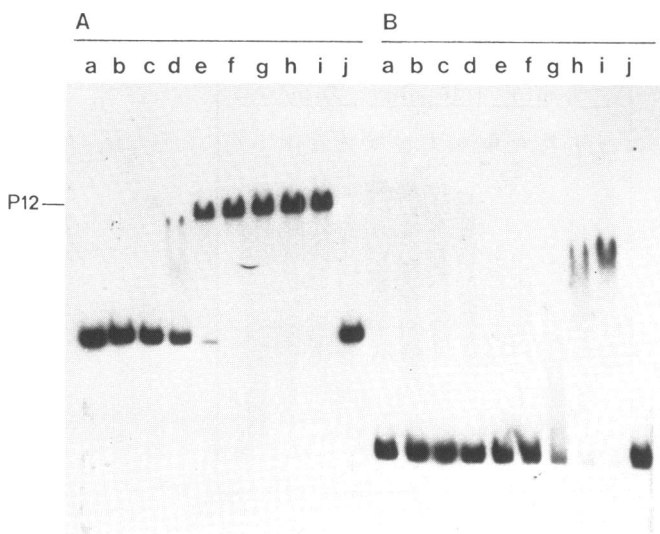
**Figure 2.** Gel retardation assay of the binding of protein P12 to ssDNA and dsDNA. The DNA fragment used in the assay was the terminal 315 bp fragment from the left end of the PRD1 genome. Binding to ssDNA is shown in panel A and binding to dsDNA in panel B. The protein amounts used were 0 ng in lanes a) and j); 15 ng in b); 30 ng in c); 75 ng in d); 150 ng in e); 300 ng in f); 750 ng in g); 1.5μg in h); and 2.5μg in i). The position of protein P12 is indicated on the left.
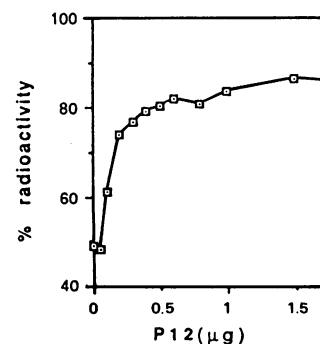
**Figure 3.** Protection of ssDNA by protein P12 against nuclease P1. The DNA fragment used in the reaction was the heat-denatured 315 bp left terminal fragment from PRD1. The percent of radioactivity indicates the proportion of trichloroacetic acid-precipitated material in the sample after nuclease treatment in the presence of different amounts of P12.
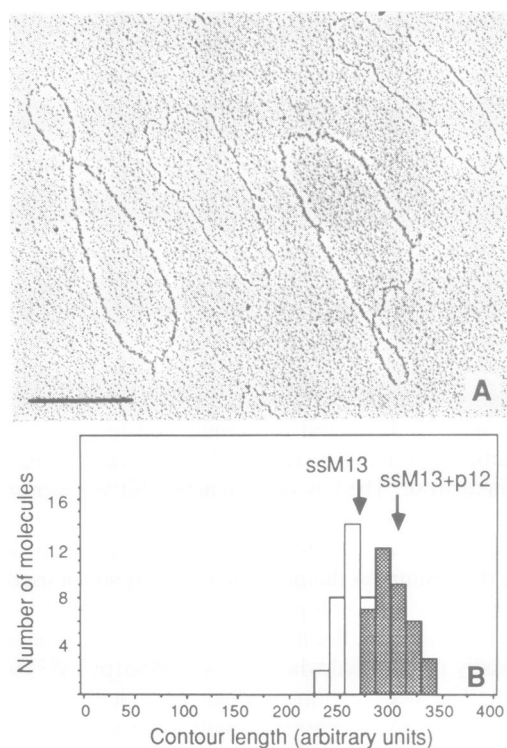
**Figure 4.** Complexes of protein P12 with ssM13 DNA. A) Structure of the complexes. Purified P12 was incubated with M13 ssDNA. DNA-protein complexes were purified and visualized under the electron microscope as described in Material and Methods. M13 ssDNA molecules, used as an internal control, are also shown. The bar represents $0.5\mu m$ (1215 nt). B) Contour length measurements of protein-free M13 ssDNA (white histogram) and P12-DNA complexes (shadowed histogram). Arrows point to the mean values of each size distribution (268.3 and 306.3 units for protein-free DNA and complexes, respectively). A total of 37 molecules were measured in each case (50 units correspond to 1359 nt)

the input ssDNA, suggesting that P12 binds to ssDNA in a cooperative way. In addition, P12 was able to retard dsDNA at a minimal protein concentration 10 times greater than that required for retarding ssDNA. The retarded dsDNA bands were more diffuse than the retarded ssDNA band, suggesting that complexes between dsDNA and P12 are not as uniform and/or stable as those formed with ssDNA. Furthermore, P12 was able to protect ssDNA against nuclease digestion (Fig. 3). Maximal protection against nuclease P1 was reached with approximately the same amount of protein that was needed for P12-ssDNA complex formation in the gel retardation assay. Binding of P12 to DNA is not sequence specific since it binds to M13 ssDNA (see below), and it retards dsDNA fragments from plasmid pUC18 with the same efficiency as the terminal PRD1 fragment (results not shown). These DNA binding properties of P12 resemble those of adenovirus DBP that also binds to both ssDNA and, with lower efficiency, to dsDNA (15,16). On the contrary, binding of the $\phi29$ SSB protein p5 to dsDNA is barely detectable (17).

**Electron microscopy of P12-ssM13 DNA complexes**

Since protein P12 binds preferentially to ssDNA it was interesting to analyze the structure of such DNA-protein complexes by direct observation under the electron microscope. Under the conditions used, P12 efficiently associates with M13 ssDNA, covering completely the DNA molecule (Fig. 4A). DNA-protein complexes had a smoothly contoured appearance, similar to that found for complexes formed between M13 ssDNA and $\phi29$ SSB protein p5 (18) or *E. coli* SSB at a high SSB to DNA ratio (35). Interaction of SSB proteins with ssDNA frequently leads to a change in the contour length of the corresponding protein-free ssDNA molecule (36). Contour length of both protein-free M13 ssDNA and P12-M13 ssDNA complexes found in the same grid was measured. In both cases, the length distributions fitted to
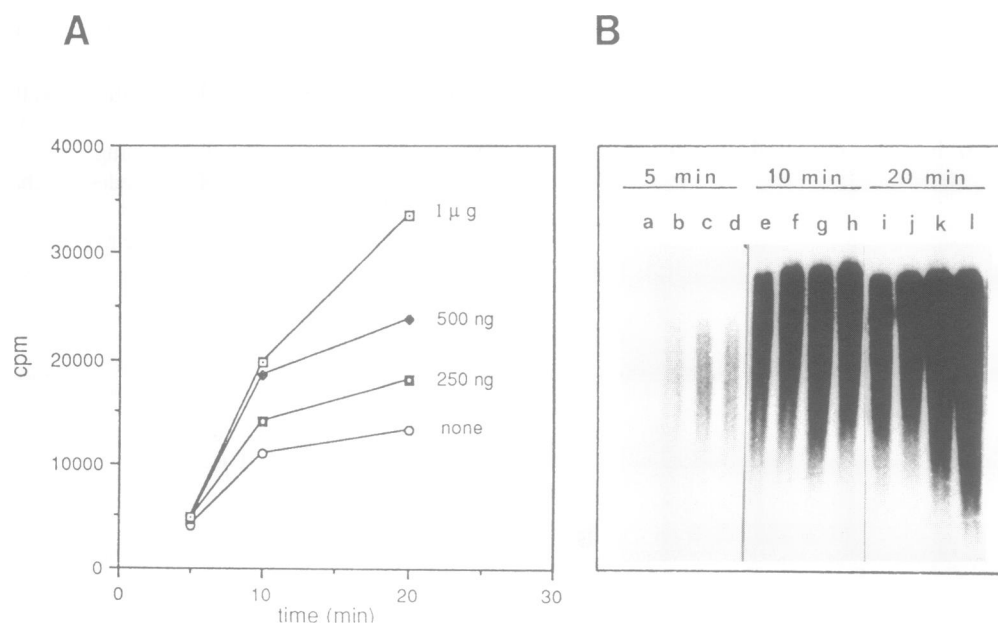


**Figure 5.** The stimulation of $\phi29$ DNA replication by P12. A) 29 DNA template was incubated with purified $\phi29$ DNA polymerase, 29 terminal protein and $40\mu M$ dNTP, in the absence or presence of P12, and the samples were processed as described in Materials and Methods. The amount of radioactivity incorporated during the time course of replication is shown in the graph. The amount of P12 used is indicated beside each curve. B) To determine the size of newly synthesized DNA products, samples from the replication assay shown in panel A were analyzed in alkaline agarose gel electrophoresis. Samples after 5 min, 10 min and 20 min replication are shown in lanes a) to d), e) to h), and i) to l), respectively. The amounts of P12 used were 0 ng in lanes a), e) and i); 250 ng in lanes b), f) and j); 500 ng in lanes c), g) and k); and $1\mu g$ in lanes d), h) and l).

dATP    1 μM   5 μM   10 μM   20 μM   40 μM   60 μM
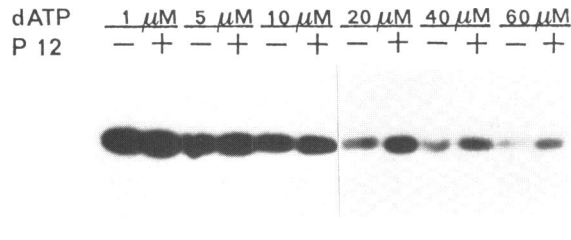P 12    − + − + − + − + − + − +

**Figure 6.** The effect of P12 on the initiation of φ29 DNA replication. Initiation reactions were carried out at different dATP concentrations, in the absence (−) or presence (+) of 2.5μg of P12, and the samples were analyzed in SDS-PAGE after 10 min of reaction.

a Gaussian curve (Fig. 4B). Comparison of the mean values indicated that interaction between P12 and M13 ssDNA produced about 14% increase in the DNA length. Although SSB proteins usually decrease the ssDNA contour length upon interaction, a 40% increase in the length of ssDNA has been also reported after binding of T4 gp32 to φX174 ssDNA (37).

### Stimulation of φ29 DNA replication by protein P12

Phage PRD1 replicates by a protein-priming mechanism similar to that of phage φ29 (for review see 9). Since an *in vitro* DNA replication system with purified components for PRD1 is not yet available, the effect of protein P12 was assayed in the *in vitro* φ29 DNA replication system (38). φ29 DNA replication was measured using φ29 terminal protein, φ29 DNA polymerase, and φ29 DNA-terminal protein complex as template (Fig. 5A). As previously described for this minimal system, DNA replication reached a plateau after 10 min (17). In the presence of protein P12, DNA replication was stimulated in a dose-dependant manner (Fig. 5A). About a 3-fold stimulation was obtained with 2.5μg of protein P12 at 40 min, when the plateau was reached (results not shown). When the DNA replication products were analyzed by alkaline agarose gel electrophoresis, it could be seen that the average length of the replication products were roughly the same both in the absence or presence of protein P12. (Fig. 5B). Therefore, it seems that protein P12 does not stimulate significantly the rate of elongation, but rather it increases the amount of molecules replicated. A similar effect has been shown for the φ29 SSB protein p5 (17). φ29 SSB protein p5 stimulates φ29 DNA replication mainly by increasing the ability of the DNA polymerase to reinitiate DNA replication, overcoming the inhibition due to the ssDNA formed during the reaction (18). Therefore, it is likely that the same mechanism operates in the case of P12.

When the effect of protein P12 on the initiation of φ29 DNA replication was studied using dATP as the only dNTP present in the incubation mixture, no stimulation of the terminal protein-dAMP initiation complex was observed up to 10μM dATP. However, about 2-fold stimulation was obtained at higher dATP concentrations (Fig. 6). Since transition from initiation to the first elongated products, terminal protein-(dAMP)$_3$, only takes place at high dATP concentrations (39), P12 likely affects this step in the initiation reaction. This stimulation could be related to the ability of P12 to bind dsDNA. The relevance of this stimulation to the overall DNA replication process remains to be determined.

### ACKNOWLEDGEMENTS

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