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Overproduction and purification of protein P6 of *Bacillus subtilis* phage ϕ29: role in the initiation of DNA replication

Ricardo Pastrana, José M.Lázaro, Luis Blanco, Juan A.García, Enrique Méndez[1] and Margarita Salas

Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, and [1]Servicio de Endocrinología, Centro Ramón y Cajal, Carretera de Colmenar Viejo, km 9, 100, 28034 Madrid, Spain

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ABSTRACT

A Ø29 DNA fragment containing gene 6, required for DNA replication, has been cloned in plasmid pPLc28 under the control of the P_L promoter of phage lambda. A polypeptide with an electrophoretic mobility close to that of p6 was labelled with ^{35}S-methionine after heat induction. This protein, representing about 4% of the total E. coli protein after 1 h of induction,was obtained in a highly purified form. The protein was characterized as p6 by amino acid analysis and NH_2-and COOH-terminal sequence determination. Protein p6 has an apparent molecular weight of 23,600, suggesting that the native form of the protein is a dimer. The purified protein p6 stimulated the protein-primed initiation of Ø29 DNA replication when added to purified proteins p2 (Ø29-coded DNA polymerase) and p3 (terminal protein).

INTRODUCTION

The Bacillus subtilis phage Ø29 has a linear, double-stranded DNA of about 18,000 base pairs (1) with a protein covalently linked to the two 5' ends (2-5). The terminal protein is the product of the viral gene 3 (2) and is required for the initiation of Ø29 DNA replication (6) that starts from either DNA end (7-9) 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. This reaction is dependent on Ø29 DNA-protein p3 complex as template (10-13) and it is catalyzed by the product of the viral gene 2(14-16) which has been shown to be a Ø29-specific DNA polymerase (17-18). The initiation reaction is strongly stimulated by some factor(s) from the host cell (18).

In addition to the viral genes 2 and 3, involved in the formation of the p3-dAMP initiation complex, three other viral genes, the products of genes 5,6 and 17 are also required for

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ø29 DNA replication *in vivo* (19,20). By *in vivo* shift-up experiments using *ts* mutants, the product of genes 5 and 6 has been shown to be involved in an elongation step in ø29 DNA replication (6). By using extracts of *B. subtilis* infected under restrictive conditions with mutants in genes 5,6 and 17, it has been shown that these gene products are not essential for the initiation reaction *in vitro* (14,15). Genes 2,3 and 5 have been cloned under the control of the P_L promoter of phage lambda (16,21) and proteins p2 and p3, which are overproduced, have been highly purified (18,22).

In this paper we describe the cloning of a ø29 DNA fragment containing gene 6 in plasmid pPLc28 (23) under the control of the P_L promoter of phage lambda in order to overproduce the protein for purification. One hour after heat induction to inactivate the *ts* cI857 repressor carried in the lambda lysogen *E.coli* strain, protein p6 represents about 4% of the total *E. coli* protein. The protein synthesized in *E. coli* has been highly purified from the induced cells and it was characterized as p6 by amino acid analysis and NH_2-and COOH-terminal sequence determination. Protein p6 stimulated the formation of the p3-dAMP initiation complex when added to purified terminal protein and ø29-coded DNA polymerase.

MATERIALS AND METHODS

Bacterial strains, plasmids and phage

The *E. coli* λ lysogen K-12Δtrp (λN⁻CI857ΔH1) (K-12ΔH1Δtrp) (24) was obtained from M. Zabeau. The *E. coli* lysogen N99 (λ^+) was obtained from M. Rosenberg (25). Plasmids pPLc28 (23) and pPLc28 lig 8 (26) were obtained from E. Remaut. *B. subtilis* 110NA try⁻ *spo*A⁻ and the ø29 mutant *sus*6(626) were as described (27). The ø29 mutant *sus*14(1242), that produces a delayed lysis phenotype, was as described (28). Minicells were prepared from *B. subtilis* CU403 thyA thyB metB div IV B1 (29) as described (30).

Enzymes

Restriction endonucleases and polynucleotide kinase were from New England Biolabs and were used according to the supplier; alkaline phosphatase from calf intestine and *E. coli* DNA polymerase I, holoenzyme or Klenow fragment, were from Boehringer

Manheim, DNAase I was from Worthington, carboxypeptidase Y from Sigma and proteinase K from Merck. T4 DNA ligase was purified by J.M. Lázaro from *E. coli* cells harbouring the plasmid pPLc28 lig 8.

DNA preparations and transformation

Plasmid DNA and DNA from the Ø29 mutant *sus*14(1242) were isolated as described by García et al. (21). Proteinase K-treated Ø29 DNA, labelled with ^{3}H-uracil, was prepared as described by Mellado et al. (6).

The Ø29 DNA restriction fragment Hind III H was isolated by electrophoresis on 3.5% acrylamide in 0.1 M Tris-borate, pH 8.3, 2 mM EDTA (TBE). The fragment was digested with Hinf I and the 440 bp long fragment containing the complete sequence of gene 6 was separated from the other ones by electrophoresis on 3.5% acrylamide. The cohesive ends of the gene 6-containing fragment and of Hind III-digested pPLc28 were filled with the Klenow fragment of DNA polymerase I and blunt-end ligated with T4 DNA ligase. Transformation of competent *E. coli* cells and detection of colonies harbouring recombinant plasmids by hybridization to ^{32}P-labelled Ø29 DNA was carried out as described (21). The recombinant plasmids containing Ø29 DNA sequences were further screened by restriction analysis.

Labelling of the DNA restriction fragments and sequencing

The gene 6-containing recombinant plasmid pRP8 was cut with Eco RI and labelled at the 5' ends with polynucleotide kinase and {γ-^{32}P}dATP (~3000 Ci/mmol, The Radiochemical Centre, Amersham) as described (31). After cutting with a second restriction enzyme and separation of the fragments, DNA sequencing was done essentially by the method of Maxam and Gilbert (32) with slight modifications (31).

Protein analysis in E. coli cells transformed by the recombinant plasmid pRP8

The *E. coli* K-12ΔH1Δtrp cells harbouring the recombinant plasmid pRP8 were grown at 30ºC as described (33). The cells were shifted to 42°C and 1 h after the shift samples were labelled for 10 min with 10 μCi ^{35}S-methionine (1200 Ci/mmol , The Radiochemical Centre, Amersham). Cells kept at 30°C were also labelled as a control. *B. subtilis* minicells infected with

wild-type phage Ø29, with mutant sus6(626) or kept uninfected were labelled with ^{35}S-methionine (200 µCi/ml; 1200 Ci/mmol) as described (30) as a control of the mobility of protein p6.

SDS-electrophoresis was carried out in slab gels containing a 10-20% acrylamide gradient (20). After electrophoresis the gels were dried for autoradiography. Densitometric analysis was performed on Kodalith ortofilm copies of the autoradiographs using an Optronics digital microdensitometer with 100 µm square raster connected to a Digital PDP11/45 minicomputer.

Amino acid analysis and protein sequence determination

Native or performic acid-oxidized protein p6 purified as described in the Results section from E. coli cells transformed with the recombinant plasmid pRP8, was hydrolyzed and subjected to amino acid analysis as described (34). Automatic Edman degradations to determine the amino terminal sequence of protein p6 was carried out as described (34).

To determine the carboxy-terminal amino acid sequence, native protein p6 (120 µg) in 0.1 ml of 0.1 M pyridine-acetate buffer, pH 5.5, was digested with carboxypeptidase Y with an enzyme to substrate ratio 1:60 (w:w) for 1, 5 and 15 min at 37°C as described (35). The mixture was then freeze-dried and used directly for automatic amino acid analysis.

Analytical ultracentrifugation

The samples were subjected to centrifugation in a Beckman-Spinco model E ultracentrifuge. Sedimentation velocity was measured using a rotor An-F with two channel cells and photoelectric scanner. The rotor speed was 48,000 rpm and the measurements were taken at 8 min intervals. Equilibrium ultracentrifugation was carried out essentially as described (36). An An-D rotor with 6 channel cells was used. The rotor speed was maintained at 40,000 rpm during 72 h at 14.6°C. At the begining and at the end of the run, the rotor was accelerated to check the homogeneity of the sample. Fringe patterns from interference pictures were measured in a Nikon microcomparator. The measurements were taken twice in each plate. The value of the partial specific volume for p6 (0.7306 cm^3g^{-1}) was obtained from the amino acid composition of the protein determined from the sequence of gene 6 (37,38).

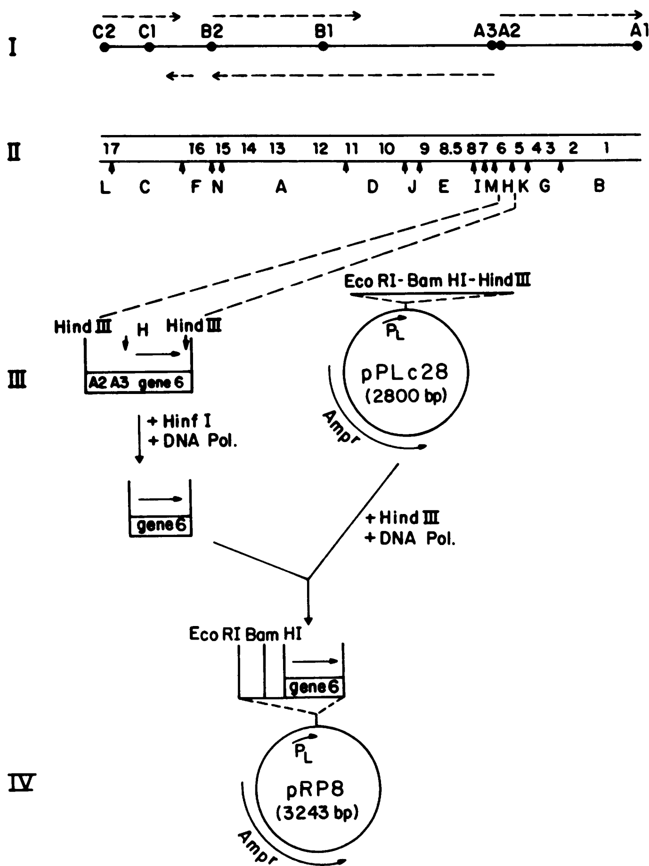

Fig. 1. Construction of the recombinant plasmid pRP8 containing the phage Ø29 gene 6 under the control of the P_L promoter of phage lambda. (I). The filled circles represent binding sites for B. subtilis RNA polymerase on Ø29 DNA and the dashed lines with the arrows the extent and direction of early transcription (top) and late transcription (bottom), drawn in opposite orientation to that described by Sogo et al. (1). (II). Genetic map of Ø29 DNA drawn in opposite orientation to that described by Mellado et al. (39). The Hind III cuts are shown below the gene numbers. (III). Enlarged Hind III H fragment showing the position of the Hinf I cuts, taken from Murray and Rabinowitz (37), by vertical arrows. The direction of transcription and the length of gene 6 and of the ampicillin gene in plasmid pPLc28 are indicated by arrows. (IV). Plasmid pRP8 showing the location of the Ø29 DNA insert and the direction of transcription of gene 6 under the control of the lambda P_L promoter.

Assay for formation of the p3-dAMP initiation complex

The incubation mixture for the initiation reaction was as described (18) using 0.25 μM {α-^{32}P}dATP (5 μCi) and purified proteins p3 (22), obtained from I.Prieto, and p2 (18) in the presence of Ø29 DNA-protein p3 as template. When indicated, purified protein p6 or extracts from uninfected B. subtilis (18) were also added. After incubation for 20 min at 30ºC the samples were processed as described (10).

Radioimmunoassay

Purified protein p6 was used to prepare anti-p6 serum. A radioimmunoassay of protein p6 was carried out as described (22).

RESULTS

Isolation and characterization of recombinants

The restriction fragment Hind III H which contains the complete sequence of gene 6 (37) as well as two Ø29 strong promoters, A2 and A3 (1), could not be cloned in the Hind III site of plasmid pPLc28, probably due to the presence of the Ø29 promoters. The fragment was cut with the restriction nuclease Hinf I giving rise to a 440 bp long fragment free of the two promoters but containing the complete sequence of gene 6 (37). As shown in Fig. 1 the fragment and plasmid pPLc28 linearized with Hind III were ligated after filling the cohesive ends with the Klenow fragment of DNA polymerase I. E. coli N99 (λ^+) was transformed with the ligation mixture, the Amp^r colonies were screened by hybridization to a ^{32}P-labelled Ø29 DNA probe and the positive clones were further checked by restriction analysis. In this way, the recombinant plasmid pRP8 with the Ø29 DNA insert in the correct orientation for transcription from the P_L promoter, was selected. The sequence of 47 nucleotides to the right and 40 nucleotides to the left of the Eco RI cut (see Fig. 1) was determined, showing to be the correct one (results not shown).

Protein synthesis directed by the recombinant plasmid pRP8

The recombinant plasmid pRP8 was used to transform the lambda lysogen E. coli strain K-12ΔH1Δtrp which has a ts cI857 mutation in the repressor gene. The cells carrying the recombinant plasmid were grown at 30ºC as described in Materials and Methods and at 1 h after induction at 42ºC they were labelled with ^{35}S-

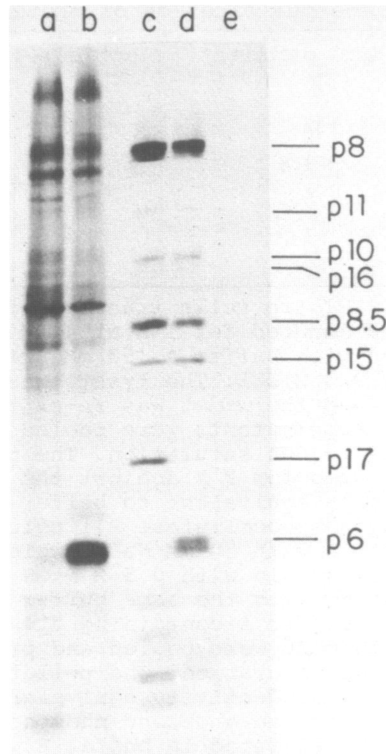

Fig. 2. SDS-polyacrylamide gel electrophoresis of extracts of cells transformed by the recombinant plasmid pRP8, labelled with ^{35}S-methionine. The E. coli K-12ΔH1Δtrp cells transformed with the recombinant plasmid pRP8 were grown at 30°C and at 1 h after the shift to 42°C the cells were labelled and subjected to electrophoresis as described in Materials and Methods (lane b). As a control, the cells transformed with plasmid pRP8 were labelled at 30°C (lane a). Lanes c-e show the electrophoresis of B.subtilis minicells infected with ø29 mutant sus6(626) (lane c), with wild-type ø29 (lane d) or uninfected (lane e), labelled with ^{35}S-methionine as described in Materials and Methods.

methionine and the proteins analyzed by SDS-polyacrylamide gel electrophoresis. Fig. 2 shows that in the cells transformed with the recombinant plasmid a polypeptide which was strongly labelled at 42ºC (lane b) was not present in the cells labelled at 30ºC (lane a). The apparent Mr of this polypeptide was ~14,000 using ^{3}H-concanavalin A peptides of known molecular weights as markers. The Mr 14,000 polypeptide had a mobility close to that of protein p6 synthesized in ø29 -infected minicells (lane d)

Table 1. Summary of the purification of protein p6

	Total protein, mg	Protein p6, %	Total protein p6, mg
Extract	132	3.6	4.7
Phosphocellulose 1	9.8	44	4.3
Phosphocellulose 2	5.1	81	4.1
Ammonium sulfate	3	95	2.8

One g of E. coli K-12ΔH1Δtrp cells transformed with the recombinant plasmid pRP8 and induced for 1 h at 42ºC was ground with alumina and extracted with buffer A (50 mM Tris-HCl, pH 7.5, 5% glycerol) containing 0.3 M KCl. The lysate was centrifuged for 10 min at 20,000 x g and the pellet was re-extracted with the same buffer. The two supernatants were pooled and precipitated with ammonium sulfate to 60% saturation. The pellet was dissolved in buffer A, dialyzed for 2 h against the same buffer, adjusted to a conductivity equivalent to buffer A with 50 mM NaCl and passed through a phosphocellulose P11 column (6.5 x 1.3 cm) equilibrated in buffer A with 50 mM NaCl, which had been regenerated by treatment for 30 min with 0.5 M NaOH and 30 min with 0.5 M HCl. After washing with the same buffer protein p6 was eluted by addition of buffer A containing 0.4 M NaCl. The fractions containing protein p6 were pooled and precipitated with ammonium sulfate to 60% saturation. The pellet was dissolved in buffer A, adjusted to a conductivity equivalent to buffer A with 10 mM NaCl and passed through a second phosphocellulose P11 column (6.5 x 1.3 cm) equilibrated in buffer A with 10 mM NaCl, which had been regenerated by treatment for 5 min with 0.5 M NaOH and 5 min with 0.5 M HCl. After washing with buffer A with 50 mM NaCl, protein p6 was eluted with buffer A containing 0.1 M NaCl. Protein p6 was finally precipitated with ammonium sulfate to 60% saturation. SDS-polyacrylamide gel electrophoresis was carried out at the different purification steps to follow the presence of protein p6. Protein concentration was determined by densitometry of the stained bands using lysozyme as standard.

which was missing in mutant sus6-infected minicells (lane c). The background in uninfected minicells was undetectable (lane e). The induced protein of Mr 14,000 accounted for about 12% of the *de novo* protein synthesis after 1 h at 42ºC.

Purification of protein p6

E. coli cells transformed with the gene 6-containing recombinant plasmid pRP8 were used as a source of protein p6 for purification. The p6 present in extracts, prepared as described in Table 1, amounts to 3.6% of the total E. coli protein after 1 h of induction at 42ºC. The extracts were passed through a column of phosphocellulose. Most of protein p6 was retained in the co-

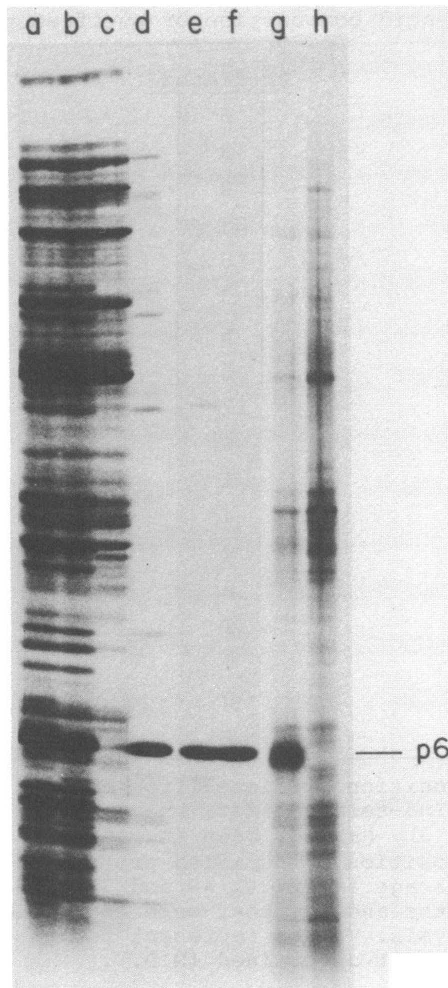

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified protein p6. Proteins at various purification steps were subjected to SDS-electrophoresis in slab gels containing a 10-20% acrylamide gradient. After electrophoresis the proteins were stained as described by Fairbanks et al. (40). (a) extract (132 µg). (b) first ammonium sulfate precipitate to 60% saturation (136µg) (c) first phosphocellulose, flow-through (76 µg). (d) first phosphocellulose, 0.4 M NaCl (10 µg). (e) second phosphocellulose, 0.1 M NaCl (5.3 µg). (f) final ammonium sulfate precipitate to 60% saturation (4.5 µg). As a marker of the electrophoretic mobility of protein p6, E. coli cells transformed with the gene 6-containing recombinant plasmid pRP8 (g) or the control plasmid pPLc28 (h) were labelled with ^{35}S-methionine at 42°C as described in Fig. 2 and, after electrophoresis, subjected to autoradiography.

Table 2. Amino acid composition of purified protein p6

Residue	Predicted[a]	Observed[b]
Asp + Asn	6	6.0
Thr	8	7.5
Ser	2	2.2
Glu + Gln	28	27.1
Pro	5	5.2
Gly	3	3.5
Ala	6	5.9
Cys	0	N.D.
Val	17	16.4
Met	6	4.4
Ile	1	1.0
Leu	4	4.1
Tyr	2	2.0
Phe	2	2.1
Trp	1	N.D.
Lys	8	8.1
His	0	0
Arg	4	4.1

[a] Amino acid composition predicted from the nucleotide sequence (37,38). The amino-terminal Met, which is removed by processing (see Table 3), has not been included.

[b] Amino acid composition of purified protein p6. Each value represents the average after 24, 48 and 72 h hydrolysis, except the values for thr and ser that were extrapolated to zero hours of hydrolysis. Values represent residues per molecule. Trp and Cys were not determined (N.D.).

lumn and eluted with 0.4 M NaCl. The protein was concentrated by precipitation with ammonium sulfate to 60% and passed through a second phosphocellulose column as described in Table 1. Protein p6 was eluted from the column with 0.1 M NaCl and finally concentrated with ammonium sulfate to 60% saturation. Figure 3 shows the analysis by SDS-polyacrylamide gel electrophoresis of the different fractions of the purification. Densitometric analysis of the protein at the final step indicated that p6 was about 95% pure. Table 1 gives a summary of the purification. About 3 mg of purified protein p6 were obtained from 1 g of E.coli

Table 3. Automatic sequential degradation of S-carboxy-methylated protein p6.

Cycle number	PTH-amino acid	Yield, nmol	Mode of identification[a]
1	Ala	6.9	A,T
2	Lys	4.9	A,T
3	Met	4.8	A,T
4	Met	2.7	A,T
5	Gln	4.5	A,T
6	Arg	3.2	A
7	Glu	4.1	A,T
8	Ile	1.4	A,T
9	Thr[b]	-	A,T
10	Lys	2.3	A,T

[a] A refers to amino acid analysis after back-hydrolysis and T to thin layer chromatography.
[b] Thr was recovered as α-aminobutyric acid after back-hydrolysis.
The amount of protein p6 used was 53 nmol.

cells harbouring the gene 6-containing recombinant plasmid pRP8.

Amino acid analysis of purified protein p6

Table 2 shows that the amino acid analysis of the purified protein p6 gave values very similar to those deduced from the DNA sequence (37,38). Moreover, as shown in Table 3, the NH_2-terminal sequence of purified protein p6, showed the sequence ala-lys-met-met-gln-arg-glu-ile-thr-lys-, in complete agreement with the one predicted from the nucleotide sequence and shown to be the starting sequence of protein p6 (37). In addition, treatment of p6 with carboxypeptidase Y sequencially released gluta-

Table 4. Amino acids released from protein p6 by treatment with carboxypeptidase Y.

Time, min	Amino acids released[a]
1	Glu(0.8),Ala(0.3),Val(0.1)
5	Glu(1.2),Ala(0.9),Val(0.5),Gln(0.3)
15	Glu(1.3),Ala(0.9),Val(0.8),Gln(0.4)

[a] Values in parenthesis are mol of amino acid/mol of protein p6

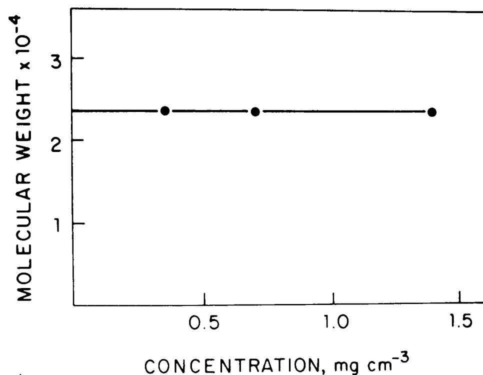

Fig.4. Molecular weight of p6 from equilibrium centrifugation analysis. Photographs were taken after reaching equilibrium at 40,000 rpm and fringe patterns measured twice in each case. The protein concentration was determined as described in the legend to Table 1. The value extrapolated to infinite dilution was obtained from the least squares straight line derived from the experimental values.

mic acid, alanine, valine and glutamine (Table 4), as expected from the nucleotide sequence (37).

Molecular weight of protein p6

Protein p6, as determined by analytical ultracentrifugation, sedimented essentially as an homogeneous sample. Figure 4 shows the apparent molecular weight of p6 obtained at different concentrations of the protein by high-speed equilibrium centrifugation. The value obtained, extrapolated to zero concentration, was 23,648.

Protein p6 stimulates the in vitro formation of the p3-dAMP initiation complex.

Figure 5 shows that a small amount of initiation complex was formed when purified proteins p2 and p3 were used (lane a). Addition of increasing amounts of purified protein p6 (lanes b-d) stimulated the initiation reaction to an extent similar to that obtained with extracts from uninfected B. subtilis (lane e), that have been shown to stimulate the formation of the initiation complex (18).

As a control that the activity stimulating the initiation reaction was intrinsic of p6 and not due to a minor contaminant, a purification was carried out in parallel with extracts from

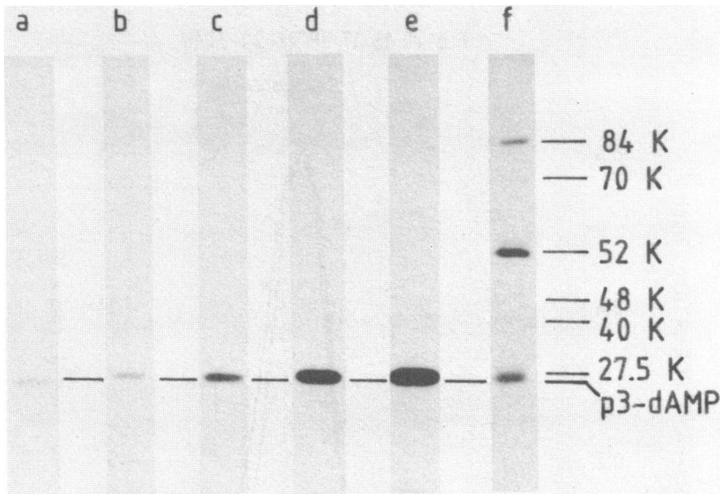

Fig. 5. Stimulation by protein p6 of the formation of the p3-dAMP initiation complex. Purified proteins p2 (10 ng) and p3 (80 ng) were incubated with ø29 DNA-protein p3 (1 µg) as template in the absence (lane a) or presence of 75 ng (lane b), 250 ng (lane c) or 750 ng (lane d) of purified protein p6 filtered through a Sephadex G50 spun-column as described (41) and the formation of the p3-dAMP complex was determined as indicated in Materials and Methods. As a control, extracts from uninfected B.subtilis (7 µg) were added (lane e). The amount of p3-dAMP formed was 0.05 (a), 0.06 (b), 0.09 (c), 0.26 (d) and 0.35 (e) fmol. Lane f, ^{35}S-labelled ø29 structural proteins.

E. coli harbouring the gene 6-containing recombinant plasmid pRP8 and from E. coli carrying the control plasmid pPLc28. Only the fractions containing protein p6, but not the control ones, stimulated the initiation reaction (results not shown). In addition,as shown in Fig. 6, when purified protein p6 was subjected to glycerol gradient centrifugation,the stimulation of the formation of the p3-dAMP initiation complex cosedimented with the position of protein p6.

DISCUSSION

The restriction fragment Hind III H, which contains the complete sequence of gene 6 (37,38) and the early and late ø29 promoters A2 and A3, respectively (1), could not be cloned in the Hind III site of plasmid pPLc28. This is probably due to the presence of the ø29 promoters since it is known that DNA fragments

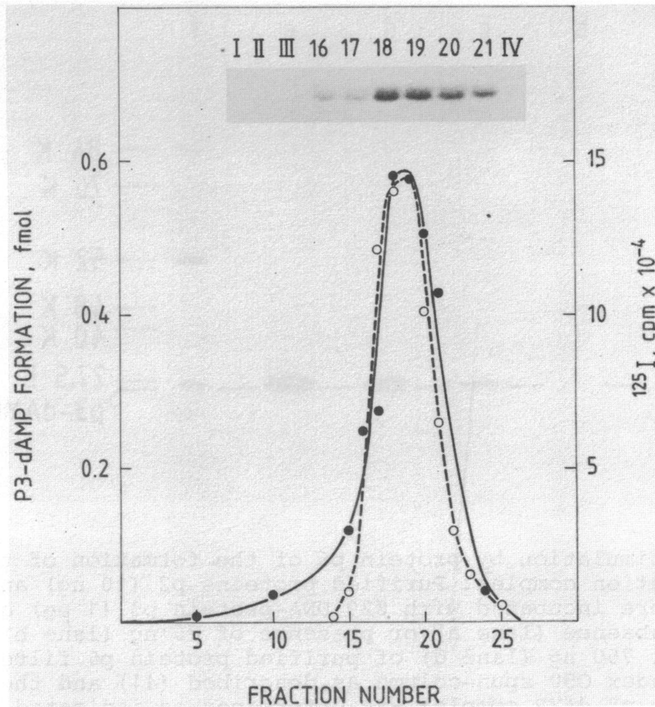

Fig. 6. Glycerol gradient centrifugation of protein p6. Purified protein p6 (200 μg) was subjected to centrifugation in a 15-30% (v/v) glycerol gradient for 21 h at 275,000 x g in a buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl. Fractions were taken and a sample from each was used to determine the position of protein p6 by radioimmunoassay (1 μl from a 1/40 dilution) as described in Materials and Methods and for the stimulation of the formation of the p3-dAMP complex by addition of 10 μl of the indicated fractions to purified proteins p2 (20 ng) and p3 (300 ng) in the presence of Ø29 DNA-protein p3 (1 μg) as template as described in Materials and Methods except that ATP was not added. The inset shows the ^{32}P-labelled p3-dAMP band. I,II,III and IV was a pool of fractions 1-5,6-10,11-15 and 22-26, respectively. Quantitation was done by excising the band from the gel and counting the Cerenkov radiation (●——●); o---o, RIA with anti-p6 serum.

containing strong promoters cannot be cloned unless strong terminators of transcription are included in the cloning vehicle (42). In support of this interpretation, we had reported that the Ø29 DNA restriction fragment Bcl I B, which also contains the A2 and A3 promoters, could not be cloned, but a fragment derived from it from which the two promoters were removed could be cloned (21). The Hind III H fragment was treated with the restriction nuclea-

se Hinf I which produces a 440 bp long fragment containing the complete sequence of gene 6, but lacking the Ø29 A2 and A3 promoters (37,1). Cloning of the above fragment in pPLc28 resulted in the recombinant plasmid pRP8, with the gene 6-containing Ø29 DNA insert in the correct orientation for transcription from the P_L promoter. After heat induction of the cells carrying the recombinant plasmid, a protein of Mr about 14,000 was labelled, accounting for about 12% of the de novo protein synthesis after 1 h. The electrophoretic mobility of this protein was close to that of protein p6 induced in Ø29-infected minicells.

We have purified protein p6 from the induced E. coli cells transformed with the recombinant plasmid pRP8. After 1 h of heat induction the amount of p6 represented approximately 3.6% of the total E. coli protein and about 3 mg of highly purified p6 was obtained from 1 g of cells. The prove that the protein p6 produced in E. coli was an intact protein was obtained by the following criteria : 1) the amino acid composition of purified p6 was very close to that deduced from the nucleotide sequence (37,38); 2) the amino- and carboxy-terminal sequence of purified p6 were identical to that predicted from the DNA sequence (37,38).

The molecular weight calculated for protein p6 from the nucleotide sequence is 11,873 (37,38). By SDS-polyacrylamide gel electrophoresis the protein had an apparent molecular weight of ~14,000, in rather good agreement with the one expected. By equilibrium centrifugation a molecular weight of 23,600 was obtained, suggesting that the native form of protein p6 is a dimer.

Protein p6 strongly stimulated the formation of the p3-dAMP initiation complex when added to purified proteins p3 (terminal protein) and p2 (Ø29-coded DNA polymerase). It was previously shown that extracts from sus6-infected B. subtilis are active in vitro in the formation of the initiation complex (14,15), suggesting that the gene 6-product is not required in the initiation reaction. This apparent contradiction with the results reported in this paper on the effect of protein p6 in initiation can be explained taking into account that the B. subtilis extracts can also stimulate the initiation of Ø29 DNA replication (18). Therefore, the extracts from sus6-infected B. subtilis were most likely providing the host factor(s) that stimulates the initiation

reaction. It remains to be determined whether, in vivo, both proteins, p6 and the host factor(s) present in the B. subtilis extracts, are needed for the initiation reaction or only one of the two proteins is required. The role of protein p6 and/or the host factor(s) in the initiation of Ø29 DNA replication could be to open the ends of the DNA and/or to keep them in an open form. Preliminary results suggest that protein p6 may be a single-stranded DNA binding protein since the purified protein binds to denatured DNA but not to native DNA. Adenovirus DNA replication that also takes place by a protein-priming mechanism, requires a viral coded single-stranded DNA binding protein involved in the initiation (43) and elongation (44) steps of replication. In vivo shift-up experiments using the Ø29 mutant ts6(1360) had suggested that the mutation can also affect an elongation process in DNA replication (6). In vitro experiments are presently being carried out to determine whether protein p6, in addition to its effect in initiation, stimulates also the elongation reaction in the replication of Ø29 DNA.

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