Volume 12 Number 5 1984 Nucleic Acids Research

Overproduction and purification of the connector protein of *Bacillus subtilis* phage ϕ29

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Received 19 December 1983; Revised and Accepted 17 February 1984

ABSTRACT

A Ø29 DNA fragment containing genes 10 and 11, coding for the connector protein and the lower collar protein, respectively, has been cloned in the pBR322 derivative plasmid pKC30 under the control of the P_L promoter of phage lambda. Two polypeptides with the electrophoretic mobility of proteins p10 and p11 were labelled with ^{35}S-methionine after heat induction. The proteins were characterized as p10 and p11 by radioimmunoassay and they represented about 10% and 7%, respectively, of the total E. coli protein after 4 hours of induction. These proteins represent less than 1% of the B. subtilis protein in Ø29-infected cells. Protein p10 has been highly purified from the E.coli cells carrying the recombinant plasmid. Antibodies raised against the purified protein p10 reacted with the connector protein produced in Ø29-infected B. subtilis.

INTRODUCTION

The head-tail connecting region in bacteriophages contains a protein, the connector, that plays a key role in the early steps of prohead assembly. It has been shown recently in the B. subtilis phage Ø29 (1,2) and in the E. coli phages T4 (3) and lambda (4) that the connector is an oligomer of a single protein with 12-fold symmetry. This is, therefore, the region at which the symmetry transition between the lower corner of the phage head (5-fold symmetry) and the tail (6-fold symmetry) occurs. According to Hendrix (5), the connector is also an active device moving the DNA into the phage prohead. In addition, in phage Ø29, the connector is essential for the normal prohead elongation (6,7).

Neck-tail complexes of phage Ø29, composed of proteins p10 (upper collar or connector), p11 (lower collar), p12* (neck appendages) and p9 (tail), have been isolated and the symmetry of the neck region (proteins p10 and p11) determined (1,2). To study the structure of the isolated connector protein, p10, and to

correlate it with that of the protein assembled in the phage necks, as well as to determine whether the protein has some catalytic activity that may be relevant to the process of DNA packaging we undertook the purification of the protein.

In this paper we describe the cloning of genes 10 and 11 in plasmid pKC30 (8) under the control of the P_L promoter of phage lambda. Four hours after heat induction protein p10 (the connector) represents about 10% of the total *E. coli* protein whereas it amounts to less than 1% of the *B. subtilis* protein in Ø29-infected cells. The connector protein synthesized in *E. coli* has been isolated in a highly purified form and antibodies against the purified protein react with protein p10 synthesized in Ø29-infected *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains, phage and plasmids

The *E. coli* strain N99 (λ^+) and plasmid pKC30 were obtained from M. Rosenberg (8). The *E.coli* λ lysogen K-12Δtrp ($\lambda N^- cI857$ Δ H1) (K-12ΔH1Δtrp) (9) was obtained from M. Zabeau. *B. subtilis* 110NA $try^- spo\ A^-$ was as described by Moreno et al. (10). The Ø29 mutant *sus*8(50011)*sus*14(1242) was as described by García et al. (11). The mutation *sus*14(1242) produces a delayed lysis phenotype (12).

Enzymes

Restriction endonucleases were from New England Biolabs and were used according to the supplier. The DNA ligase and nuclease Bal 31 were from Bethesda Research Laboratories, *E.coli* DNA polymerase I from Boehringer, DNase I from Worthington and fungal proteinase K from Merck.

DNA preparations and transformation

Plasmid and phage Ø29 DNA were isolated as described by Clewell and Helinski (13) and Inciarte et al. (14), respectively. The Ø29 DNA fragment Pvu I A (see Fig. 1) was isolated by centrifugation in a 5-20% sucrose gradient as described by Salas et al. (15). About 4 µg of the fragment were digested with 4 units of nuclease Bal 31 for 3 min at 30°C in a buffer containing 20 mM Tris-HCl, pH 8.1, 12 mM $CaCl_2$, 1 mM EDTA and 600 mM NaCl. After phenol extraction and ethanol precipitation the Bal

31-treated Pvu I A fragment (2 µg) was blunt-end ligated to Hpa I-linearized plasmid pKC30 (3 µg) by incubation with T4 DNA ligase as described by García et al. (16).

Transformation of competent E. coli cells and detection of colonies harbouring recombinant plasmids by hybridization to ^{32}P-labelled Ø29 DNA were carried out as described (16). The recombinant plasmids containing Ø29 DNA sequences were further screened by restriction analysis to determine the size and the orientation of the insert.

Protein analysis in cells transformed by the plasmids

The cells were grown at 30°C in LB medium with ampicillin (50 µg/ml). At A_{420} = 0.46 the bacteria were collected by centrifugation and resuspended in the same volume of minimal media (17). After 1 h at 30°C the temperature was shifted to 42°C and, at the indicated times, samples of 0.05 ml were labelled for 10 min with 10 µCi of ^{35}S-methionine (1200 Ci/mmol, The Radiochemical Centre, Amersham). When indicated, after the 10 min pulse a 1000 fold excess of cold methionine was added and samples removed after different times. Samples were subjected to SDS-polyacrylamide gel electrophoresis as described below.

To determine protein accumulation the bacteria were grown in LB medium and samples were taken at different times after the shift at 42°C. The bacteria were concentrated 10-fold by centrifugation and resuspension in a buffer containing 100 mM Tris-HCl, pH 8.0, 200 mM KCl, 1 mM EDTA, 2 mM $CaCl_2$, 10 mM $MgCl_2$, and 5% glycerol and lysed by sonication. Bacterial debris were removed by centrifugation for 5 min at 8900 g and the samples subjected to SDS-polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis

SDS-electrophoresis was carried out either in slab gels containing 10% acrylamide (18) or in 10-20% acrylamide gradients (12). For the radioimmunoassay of protein p10 after electrophoresis, 4 M urea was present both in the stacking and the separation gels. The gels were either stained as described by Fairbanks et al. (19) or dried for autoradiography. Densitometric analysis was performed on the autoradiographs or on Kodalith orthofilm copies of the stained gels, using an Optronics digital microdensitometer with a 100 µm square raster, connected to a Digital

PDP 11/45 minicomputer.

Preparation of sera

The neck-tail complexes of Ø29, containing proteins p9,p10, p11 and p12* were isolated from phage particles as described by Carrascosa et al. (1). Protein p10 was purified from E. coli containing the recombinant plasmid pKC30 X16 as described in this paper.

Rabbits were immunized with two 100 µg doses of total protein, the first time in complete Freund's adjuvant. Serum was collected one week after the last injection.

Characterization of proteins p10 and p11 by radioimmunoassay

Cellular extracts of E. coli transformed with the recombinant plasmids prepared as described above were subjected to SDS-polyacrylamide gel electrophoresis and proteins p10 and p11 were detected by radioimmunoassay carried out as described (16) using anti neck-tail serum and ^{125}I-protein A (a gift from I. Prieto).

B. subtilis 110NA was infected with the Ø29 mutant sus8(50011) sus14(1242) and labelled with ^{35}S-sulfate (200 µCi/ml; 25-40 Ci/mg, The Radiochemical Centre, Amersham) as described by Camacho et al. (20). After 60 min at 37°C the cells were lysed with lysozyme, treated with DNase I and RNase A and the cell debris were removed by low-speed centrifugation. A control of uninfected bacteria was labelled and processed in the same way. A radioimmunoassay was carried out following the procedure developed by J.A. Melero (personal communication). Briefly, protein A is bound to wells of a polystyrene multiwell plate followed by incubation first with anti-protein p10 serum and then with the ^{35}S-labelled extracts of B. subtilis infected with mutant sus8 (50011)sus14(1242). The bound protein is analyzed by SDS-polyacrylamide gel electrophoresis.

RESULTS

Isolation and characterization of recombinants

The restriction fragment Pvu I A containing the late region of Ø29 DNA, transcribed from left to right (see Fig. 1), was digested under controlled conditions with nuclease Bal 31 and blunt-end ligated to plasmid pKC30 linearized with Hpa I (8). E. coli N99 (λ^+) was transformed with the ligation mixture, the amp^r

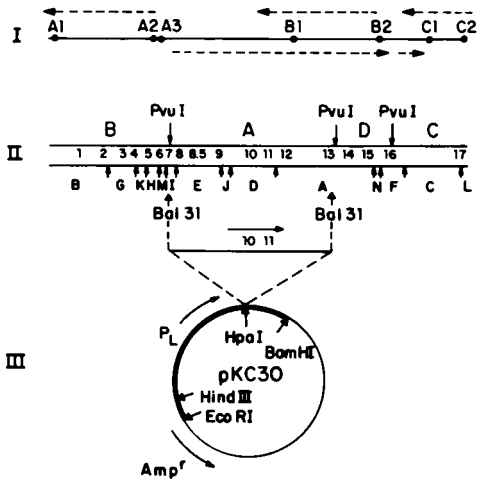

Fig.1. Construction of pKC30 recombinant plasmids containing the Ø29 DNA fragment Pvu I A digested with Bal 31. (I) The filled circles represent binding sites for B. subtilis RNA polymerase on Ø29 DNA and the dashed lines with the arrows the extent and direction of early transcription (top) and late transcription (bottom) as described by Sogo et al. (21). (II) Genetic map of Ø29 DNA taken from Mellado et al. (22). The location of the Pvu I and Hind III cuts has been taken from Yoshikawa and Ito (23). The Hind III cuts are shown below the gene numbers with small arrows. (III) Plasmid pKC30 showing the location of the Ø29 DNA insert. The direction of transcription of the ampicillin gene and of the Ø29 genes 10 and 11 from the lambda P_L promoter are indicated by arrows.

colonies were screened by hybridization to a ^{32}P-labelled Ø29 DNA probe and the positive clones were further checked by Hind III digestion. Several recombinant plasmids containing fragment Hind III D were obtained, indicating the insertion of the Bal 31-digested Pvu I A fragment (see Fig. 1). Insertions of a wide range of sizes were obtained; in addition, Bal 31 digested asymmetrically from each end of the Pvu I A fragment. Treatment of the above recombinant plasmids with Hind III produced, besides the Ø29 DNA Hind III D fragment, two other ones corresponding to the fusion of the rest of the Ø29 DNA inserted and each of the two pKC30 fragments of 4.7 and 1.6 kb which are present between the Hind III site of the plasmid and the Hpa I insertion site (see Fig. 1). The analysis of these fragments showed that in the recombinant plasmid pKC30 X16 the Ø29 DNA insert (~5.8 kb) was in

the correct orientation for transcription from the P_L promoter and in plasmid pKC30 X6 the insert (~5.9 kb) was in the opposite orientation. Therefore, Bal 31 had digested about 100 and 2500 nucleotides, respectively, from the right and left ends of the Pvu I A fragment in plasmid pKC30 X16 and about 300 and 2200 nucleotides, respectively, from the right and left ends of the fragment in plasmid pKC30 X6.

Protein synthesis directed by the recombinant plasmids

The recombinant plasmids were used to transform the lysogen E. coli strain K-12ΔH1Δtrp with the thermosensitive cI857 mutation in the lambda repressor. By raising the temperature from 30° to 42°C the repressor is inactivated allowing the expression of the genes under the control of the lambda P_L promoter. The above cells, carrying the recombinant plasmids pKC30 X16 or pKC30 X6, were grown at 30°C as described in Materials and Methods and at 30 min, 60 min and 120 min after induction at 42°C, they were labelled with ^{35}S-methionine and the proteins analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2, in the cells transformed with the recombinant plasmid pKC30 X16, with the Ø29 DNA insert in the correct orientation for transcription from the P_L promoter, one polypeptide with the same electrophoretic mobility as the Ø29 protein p10 was clearly labelled after 60 or 120 min at 42°C. This polypeptide was not present in the cells transformed with the control plasmid pKC30. In the case of plasmid pKC30 X6, with the Ø29 DNA inserted in the opposite orientation, the same polypeptide was labelled, although in smaller extent, suggesting that a Ø29 promoter could be used to transcribe this gene in the plasmid (see later). At 30°C, the synthesis of this protein was much smaller than at 42°C in the cells transformed by either recombinant plasmid, pKC30 X16 or pKC30 X6.

A polypeptide with the electrophoretic mobility expected for protein p11 was also labelled after heat induction in the cells transformed with both recombinant plasmids, pKC30 X16 and pKC30 X6, although some protein of similar size was also labelled in the cells transformed with the control plasmid pKC30 (see Fig. 2). The evidence that the protein labelled in the cells transformed by the recombinant plasmids was, indeed, p11, was obtained by radioimmunoassay (see later).

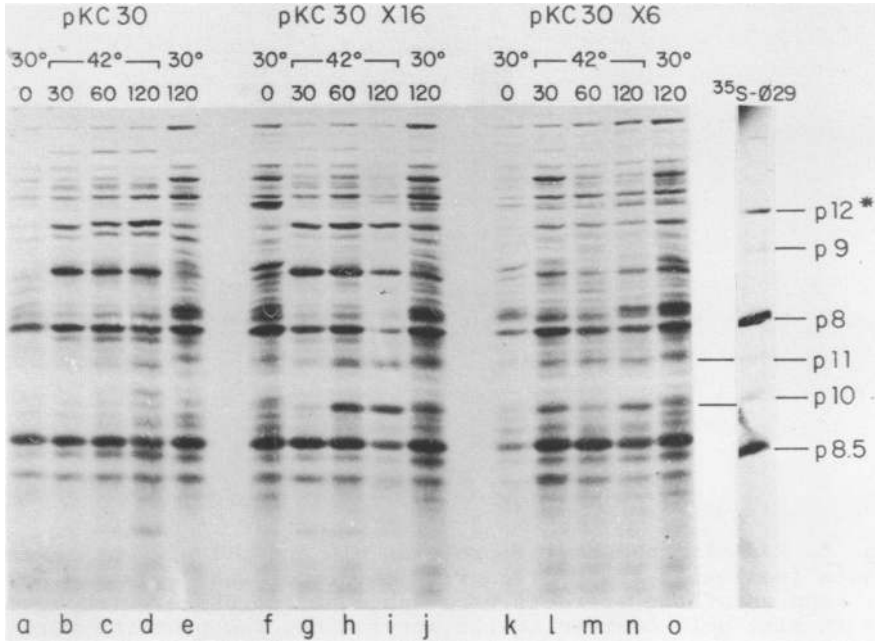

Fig. 2. SDS-polyacrylamide gel electrophoresis of extracts of cells transformed by the recombinant plasmids, labelled with ^{35}S-methionine. The cells transformed with the recombinant plasmids pKC30 X16 or pKC30 X6 or with the control plasmid pKC30 were grown at 30°C and at 30, 60 and 120 min after the shift to 42°C, labelled and subjected to electrophoresis in 10% acrylamide gels as described in Materials and Methods. As a control, cells kept at 30°C were labelled at the time of induction and 120 min later. The starting time of each labelling period (in min) and the temperature of incubation are indicated above the slots. ^{35}S-labelled Ø29 structural proteins were used as molecular weight markers (24).

Characterization of proteins p10 and p11 by radioimmunoassay

To determine whether the extracts of *E. coli* transformed with the recombinant plasmids contained indeed proteins p10 and p11, cells were induced at 42°C and, after 120 min, extracts were subjected to SDS-polyacrylamide gel electrophoresis and the proteins transferred to two cellulose nitrate papers. One of them was stained and the other one incubated first with anti-Ø29 neck-tail serum and then with ^{125}I-protein A. Autoradiography showed the presence of labelled bands at the position of proteins p10 and p11 in the extracts from cells transformed by the recombi-

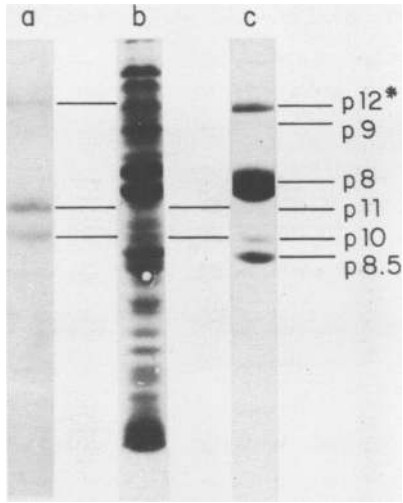

Fig. 3. Radioimmunoassay of proteins p10 and p11 present in extracts from cells transformed with the recombinant plasmid pKC30 X6. Samples of the extracts were subjected to SDS-electrophoresis in slab gels containing 10% acrylamide. The proteins were transferred from the gel to two sheets of nitrocellulose paper which were incubated with anti-neck-tail serum and ^{125}I-protein A (a) or stained with amido black (b), respectively, as described in Materials and Methods. c, structural proteins of Ø29.

nant plasmid pKC30 X6 (Fig. 3a) or pKC30 X16 (not shown). In addition, a band with the electrophoretic mobility of p12* was also labelled in the cells transformed by the recombinant plasmid pKC30 X6, suggesting the presence of gene 12 in the above recombinant plasmid. This is in agreement with the fact that Bal 31 digestion had only removed 300 nucleotides from the right end of fragment Pvu I A present in the recombinant plasmid pKC30 X6. Protein p10 was detected by staining but very low amount of protein p11 was present in the extracts of the transformed cells (Fig. 3b) due to the fact that protein p11 sedimented after low speed centrifugation (see later).

Stability of proteins p10 and p11

Proteins p10 and p11 induced in E. coli harbouring plasmid pKC30 X16 were synthesized at a high rate at least up to 2 h after heat induction (see Fig. 2). To test the stability in E.coli of the induced proteins, cells transformed with plasmid pKC30 X16 were shifted to 42°C, induced for 75 min, incubated 10 addi-

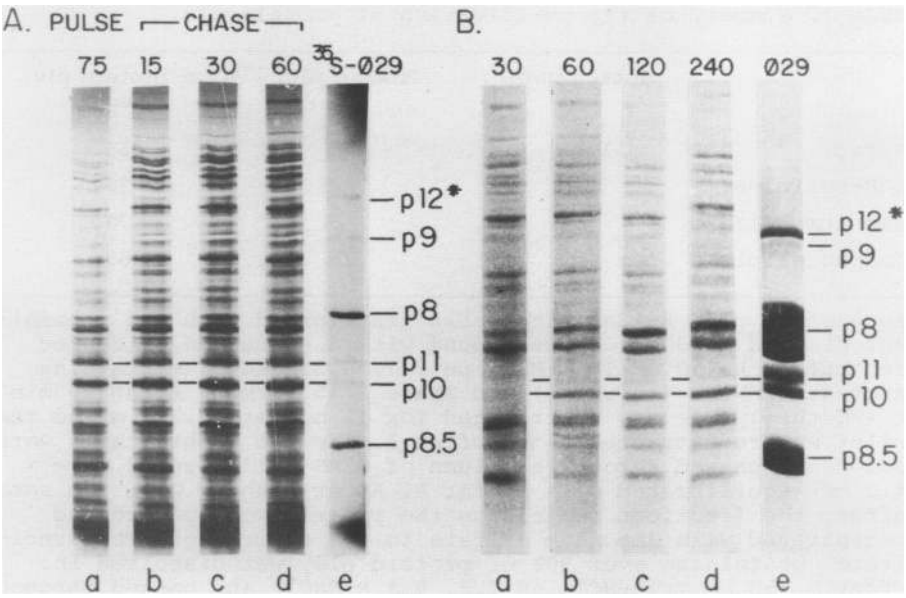

Fig. 4. Stability of proteins p10 and p11. A. E. coli cells carrying the recombinant plasmid pKC30 X16 were shifted to 42°C and, after 75 min, labelled with ^{35}S-methionine for 10 min (a). The labelled proteins were chased by addition of a 1000 fold excess of cold methionine and samples removed at 15 min (b), 30 min (c) and 60 min (d) and subjected to SDS-electrophoresis in 10% acrylamide slab gels. ^{35}S-labelled Ø29 structural proteins were used as molecular weight markers in (e). B. E. coli cells carrying plasmid pKC30 X16 were induced at 42°C and at 30 min (a), 60 min (b), 120 min (c) and 240 min (d) samples were removed and the extracts, prepared as described in Materials and Methods, were subjected to SDS-electrophoresis in 10% acrylamide slab gels. Ø29 structural proteins were used in (e) as molecular weight markers. After electrophoresis the gel was subjected to autoradiography (A) or stained (B) as described in Materials and Methods.

tional min in the presence of ^{35}S-methionine and the labelled polypeptides chased by addition of an excess of cold methionine. Figure 4A shows that the two proteins, with the electrophoretic mobility of p10 and p11, were stable after a chase of 1 h. Taking into account the stability of the proteins and their high rate of synthesis for at least 2 h it was expected to find progressive accumulation of proteins p10 and p11 after heat induction of cells harbouring the recombinant plasmid pKC30 X16. Fi-

Table 1. Summary of the purification of protein p10.

	Total protein, mg	Protein p10, %	Total protein p10, mg
Extract	1,404	11	154
DEAE-cellulose	984	8	79
Ammonium sulfate	280	25	70
Phosphocellulose	63	95	60

Ten g of E. coli K-12ΔH1Δtrp cells transformed with the recombinant plasmid pKC30 X16 were ground with alumina and extracted with buffer A (50 mM Tris-HCl, pH 7.7, 0.3 M KCl), containing pancreatic RNase (10 µg/ml) and DNase I (5 µg/ml). After 30 min at 4°C the lysate was centrifuged for 15 min at 19,700 g and the pellet was re-extracted with buffer A. The two supernatants were pooled and passed through a column of DEAE-cellulose (3.6 cm x 24.3 cm) equilibrated with buffer A. After washing with the same buffer, the fractions containing the protein were pooled and precipitated with ammonium sulfate to 43% saturation. The precipitate, containing over 90% of protein p10, was dissolved in buffer B (50 mM Tris-HCl, pH 7.7, 0.1 M NaCl) and passed through a column of phosphocellulose (2.9 cm x 24.6 cm) equilibrated with buffer B containing 5% glycerol). The column was washed stepwise with 4 volumes of buffer B containing 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M NaCl, respectively. Protein p10 eluted at 0.6 M NaCl essentially free of contaminating proteins. SDS-polyacrylamide gel electrophoresis was carried out at the different purification steps to follow the presence of protein p10. Protein concentration was determined by the method of Bradford (25).

gure 4B shows that there was a progressive accumulation of protein p10 up to at least 4 h, representing at this time about 10% of the total E. coli protein. The amount of protein p11 was very low due to the fact that most of p11 sediments after low-speed centrifugation (see later).

Purification of protein p10

The amount of protein p10 synthesized in Ø29-infected B.subtilis represents less than 1% of the total bacterial protein (data not shown). Since in the E. coli cells transformed with the gene 10-containing recombinant plasmid pKC30 X16 protein p10 represents about 10% of the total protein after 4 h of heat induction, we used these cells as a source of protein p10 for purification. Lysates were centrifuged as described in the legend to Table 1. It can be seen in Fig. 5, lanes b and c, that most of protein p11 is lost by low-speed centrifugation of the lysate

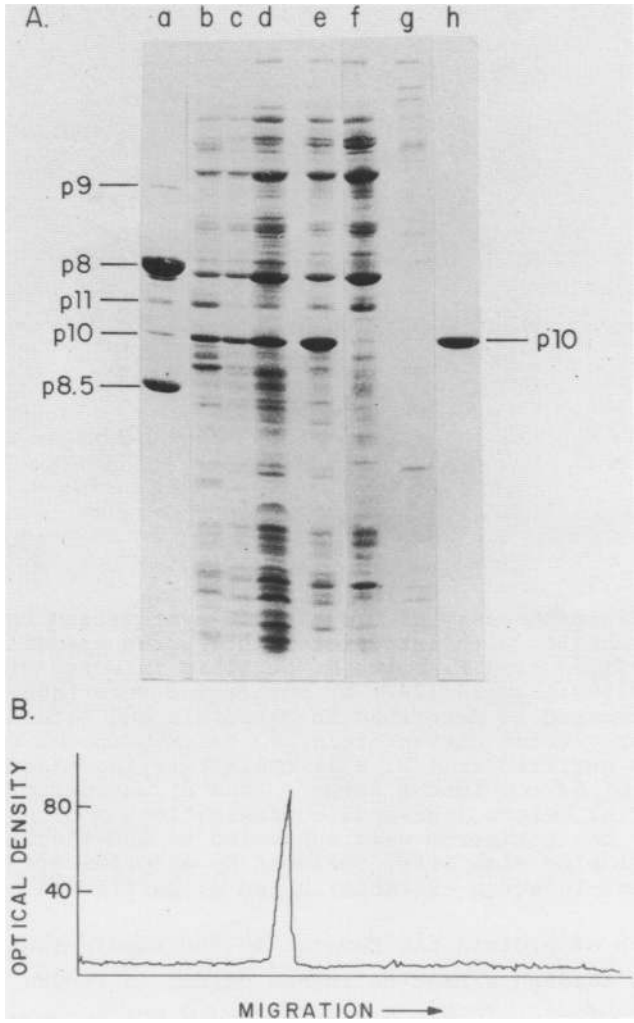

Fig.5. SDS-polyacrylamide gel electrophoresis of purified protein p10. A. Proteins at various purification steps were subjected to SDS-electrophoresis in slab gels containing a 10-20% acrylamide gradient. After electrophoresis the proteins were stained as described in Materials and Methods. (a) structural proteins of Ø29 12⁻ particles. (b) lysate (48 µg); (c) extract, (36 µg); (d) DEAE-cellulose (180 µg); (e) ammonium sulfate (140 µg); (f) phosphocellulose, flow-through (80 µg); (g) phosphocellulose,0.4 M NaCl (14 µg); (h) phosphocellulose, 0.6 M NaCl (18 µg). B.Densitometric analysis of purified protein p10 (phosphocellulose fraction).

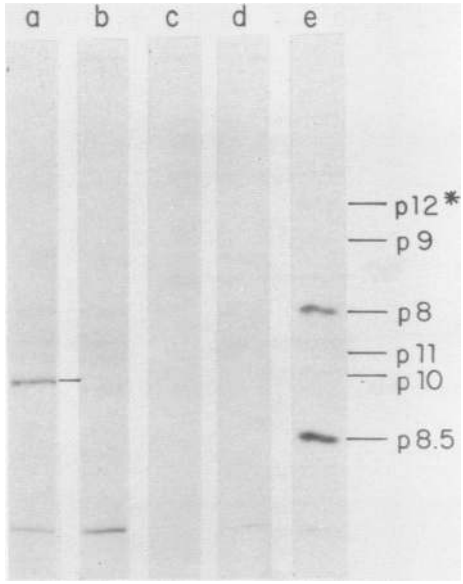

Fig. 6. Radioimmunoassay of protein p10 synthesized in Ø29-infected B. subtilis with anti-protein p10 serum prepared with the protein purified from E. coli. B. subtilis infected with the Ø29 mutant sus8(50011)sus14(1242) or uninfected were labelled and extracts prepared as described in Materials and Methods. The radioimmunoassay using anti-protein p10 serum prepared with the protein p10 purified from E. coli cells carrying plasmid pKC30 X16 (a and b) or non-immune serum (c and d) was performed as described by J.A. Melero (personal communication) and the proteins retained by the antiserum were subjected to SDS-electrophoresis in 10% acrylamide slab gels, followed by autoradiography. a and c, Ø29 mutant-infected extracts; b and d, uninfected extracts.

whereas most of protein p10 remains in the supernatant. Extracts were passed through a DEAE-cellulose column to remove nucleic acids. Most of protein p10 eluted at 0.3 M KCl and was precipitated with ammonium sulfate to 43% saturation. Protein p10 was further purified by phosphocellulose chromatography, eluting at 0.6 M NaCl essentially free of contaminating proteins (Fig. 5). Table 1 gives a summary of the purification. About 60 mg of purified protein p10 were obtained from 10 g of E. coli harbouring the gene 10-containing recombinant plasmid pKC30 X16.

Crossreaction of protein p10 synthesized in Ø29-infected B. subtilis with serum against protein p10 purified from E. coli carrying plasmid pKC30 X16

Protein p10 synthesized in E. coli containing plasmid pKC30

X16, purified as described above, was used to raise antibodies. A ^{35}S-labelled extract of B. subtilis infected with a Ø29 mutant unable to assemble heads (to get p10 in soluble form) was reacted with the above anti-p10 serum. SDS-polyacrylamide gel electrophoresis of the proteins that were bound to this serum shows that the only protein specifically retained was p10 (Fig. 6a). Non-immune serum did not react with protein p10 from Ø29-infected B. subtilis (Fig. 6c). No band with the mobility of protein p10 was obtained from uninfected B. subtilis either with anti-protein p10 serum or non-immune serum (Fig. 6b and d).

DISCUSSION

Fragment Pvu I A contains the sequence coding for proteins p8 (major head protein), p8.5 (fibers), p9 (tail), p10 (connector), p11 (lower collar), p12 (precursor of the neck appendages) and probably part of the morphogenetic proteins p7 and p13 (22, 23). After digestion of the fragment with Bal 31 the resulting mixture was cloned in the Hpa I site of plasmid pKC30 under the control of the lambda P_L promoter. Two recombinant plasmids were selected, pKC30 X16 and pKC30 X6, with the Ø29 DNA insert in the correct orientation for transcription from the P_L promoter and in the opposite one, respectively. After heat induction of the cells carrying the recombinant plasmids, two proteins with the electrophoretic mobility of p10 (connector) and p11 (lower collar) were labelled, which were synthesized in smaller extent at 30°C or in the cells transformed with the control plasmid pKC30. The phage protein p12* might be synthesized, as shown by radioimmunoassay, but no other viral protein was synthesized, in agreement with the fact that nuclease Bal 31 had cut 0.1 kb from the right end and 2.5 kb from the left end of the Pvu I A fragment inserted in plasmid pKC30 X16 and 0.3 kb from the right end and 2.2 kb from the left end of the fragment inserted in pKC30 X6. The induced proteins were characterized as p10 and p11 by radioimmunoassay. It is known that E. coli RNA polymerase can recognize Ø29 late promoters in vitro (26). The synthesis of significant levels of the two proteins, p10 and p11, in the cells transformed with the recombinant plasmid pKC30 X6, with the Ø29 DNA insert in the opposite direction of transcription from the

P_L promoter, suggests that a Ø29 late promoter or promoter-like sequence is present in the inserted fragment and could be recognized by E. coli RNA polymerase. Why the proteins with the electrophoretic mobility of p10 and p11 seem to be induced at 42°C in the cells transformed with the recombinant plasmid pKC30 X6 is not understood at present.

The two proteins, p10 and p11, synthesized in E. coli transformed with plasmid pKC30 X16, were stable for at least 1 h as shown by a pulse and chase experiment. The accumulation of the two proteins in E. coli was determined by densitometry of the stained gels after separation by SDS-polyacrylamide electrophoresis of the proteins present at different times after the shift at 42°C. The maximal amount of proteins p10 and p11 accumulated after 4 h of induction was about 10% and 7%, respectively, of the total E. coli protein. These proteins represent less than 1% of the B. subtilis protein in Ø29-infected cells.

We have purified protein p10 from E. coli cells transformed with plasmid pKC30 X16. About 60 mg of highly purified protein p10 was obtained from 10 g of cells. Antibodies raised against the purified protein recognize protein p10 induced in Ø29-infected B. subtilis. Moreover, the purified protein p10 aggregates giving rise to the same type of structures found in the neck complexes isolated from phage Ø29 particles (unpublished results). The structural as well as the functional properties of purified protein p10 are presently being studied.

ACKNOWLEDGEMENTS

We wish to thank Dr. M. Rosenberg for allowing us to use plasmid pKC30, to Dr. R.P. Mellado for his contribution in the preliminary cloning experiments and to J.M. Lázaro for growing the bacteria and help in the initial steps of the purification. This investigation has been aided by research grant 2 R01 GM27242-04 from the National Institutes of Health and by grants from the Comisión Asesora para el Desarrollo de la Investigación Científica y Técnica and Fondo de Investigaciones Sanitarias. J.A.G. is recipient of a Postdoctoral fellowship from the Spanish Research Council.

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