Regions at the carboxyl end of bacteriophage ϕ 29 protein p6 required for DNA binding and activity in ϕ 29 DNA replication

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ABSTRACT

Series of deletions corresponding to the carboxyl end of the phage ϕ 29 protein p6 have been constructed and their activity in the initiation of ϕ 29 DNA replication and their capacity to interact with the ϕ 29 DNA ends have been studied. Determination of the activity of the deletion mutants in ϕ 29 DNA replication indicated the dispensability of the 14 carboxy-terminal amino acids of the protein. The activity of protein p6 decreased with deletions from 23 to 39 amino acids and was undetectable when 44 amino acids were removed. A similar behaviour was obtained when the interaction of the mutant proteins with the ϕ 29 DNA ends was analyzed. These results indicate that the stimulation of ϕ 29 DNA replication by protein p6 requires a specific binding to the ϕ 29 DNA ends.

INTRODUCTION

The *Bacillus subtilis* phage ϕ 29 has a linear double-stranded DNA, 19,285 bp long (1) with the viral protein p3 covalently linked to each 5' end (2) through a phosphoester bond between the OH group of serine residue 232 and dAMP, the terminal nucleotide at both 5' ends (3,4). Phage ϕ 29 DNA replicates by a strand-displacement mechanism (5-7), starting at either end of the DNA-protein p3 template by formation of a p3-dAMP covalent complex in the presence of free protein p3, viral DNA polymerase p2, and dATP (8,9). The protein p3-dAMP initiation complex is further elongated by the viral DNA polymerase p2 to produce full-length ϕ 29 DNA (10).

The viral gene 6 product is needed to synthesize ϕ 29 DNA *in vivo* (11,12). Although there is not an absolute requirement for protein p6 in the *in vitro* replication system, the protein stimulates both the initiation reaction and the transition from the p3-dAMP initiation complex to the first elongated products (13). Protein p6, 103 amino acids long (14), forms dimers in solution at high protein concentration (15) and binds specifically to the ϕ 29 DNA ends forming a nucleoprotein complex that extends over the first 200-300 bp at both genome ends (16), where the replication origins are located (17). In addition, protein p6 binding changes the DNA conformation as shown by the restraining of positive supercoiling when bound to closed circular DNA (18). To define structural and functional domains and to correlate the protein p6 functions, series of deletion mutants corresponding to the carboxyl end have been constructed, overproduced in *E. coli*, highly purified and assayed for ϕ 29 DNA binding and replication activity.

MATERIALS AND METHODS*Bacterial strains and plasmids*

The *E. coli* λ lysogen K-12 Δ trp (λ N⁻cI857 Δ H1) (K-12 Δ H1 Δ trp) (19) was obtained from E. Remaut. Plasmids pRP8 (15), pRMOss and pAZe3ss (20) were used in the cloning experiments.

Enzymes and radiolabelled precursors

Restriction enzymes and nuclease Bal 31 were from New England Biolabs; Klenow fragment of *E. coli* DNA polymerase I, calf intestinal alkaline phosphatase, micrococcal nuclease and T4 DNA ligase were from Boehringer-Mannheim; DNase I was from Worthington;

[α - 32 P]dATP (approx. 400 Ci/mmol) and 35 S-methionine (1200 Ci/mmol) were from Amersham International, plc.

DNA manipulations

Plasmid DNA preparation, DNA ligation and agarose gel electrophoresis were as described (20). Elution of DNA fragments from agarose gels was as described (21). Nucleotide sequence analysis was performed by the enzymatic dideoxy chain-termination method (22). The BamHI-Sau3AI restriction fragment from plasmid pRP8 was cloned in plasmid pRMOss, which contains translation stop signals in the three possible reading frames and in the two orientations, to generate plasmid pMJc Δ 14 which lacks fourteen amino acids at the carboxyl end of protein p6. To construct plasmids pMJc Δ (23–44), lacking 23–44 amino acids at the carboxyl end of protein p6, HindIII-linearized plasmid pMJc Δ 14 was digested with Bal 31 as described (23) and cloned in plasmid pAZe3ss, which contains translation stop signals in the three possible reading frames. DNA ligation was performed in the presence of 15% PEG (24). The concatemers were digested with PstI and the vector-insert monomers were recircularised under standard ligation conditions prior to transformation.

Protein analysis in E. coli cells transformed by the recombinant plasmids

The *E. coli* K-12 Δ H1 Δ trp cells harbouring the different recombinant plasmids were grown at 30°C as described (25). The cells were shifted to 42°C and 1 h after the shift, samples were either directly subjected to SDS-electrophoresis in slab gels containing a 10–20% acrylamide gradient (26) and the proteins stained as described (27), or labelled for 2 min with 10 μ Ci of 35 S-methionine, electrophoresed as indicated above and then the gels were dried for autoradiography. Cells kept at 30°C were also processed and electrophoresed as a control.

Purification of mutant proteins

Five g of *E. coli* K-12 Δ H1 Δ trp cells transformed with the recombinant plasmid pMJc Δ 14 and induced for 1 h at 42°C were ground with alumina and purified similarly to the wild-type protein p6 (15), except that protein p6c Δ 14/+PVID (p6c Δ 14) was eluted from the first phosphocellulose column (50 ml) with buffer A (50 mM Tris-HCl, pH 7.5, 5% glycerol) + 1 M NaCl. The fractions containing protein p6c Δ 14 were pooled, dialyzed against buffer A and passed through a second phosphocellulose column (5 ml) equilibrated in buffer A + 50 mM NaCl. After washing with buffer A + 50 mM NaCl, protein p6c Δ 14 was eluted with buffer A + 0.25 M NaCl, dialyzed against buffer A and passed through a third phosphocellulose column (200 μ l) equilibrated in buffer A + 50 mM NaCl. After washing with the same buffer, protein p6c Δ 14 was eluted with buffer A + 2 M NaCl.

Fig. 1. Construction of recombinant plasmids encoding deletions at the carboxyl end of protein p6. (A) The BamHI-SaFigu3AI restriction fragment from plasmid pRP8 was placed under the control of the λ P_L promoter present in plasmid pRMOss. This plasmid contains the oligodeoxynucleotide TGATTGATTGA (20), so that the stop codon TGA is placed downstream of the deleted p6-coding sequence in any possible reading frame. (B) The HindIII site present in plasmid pMJc Δ 14 was used to linearize the plasmid prior to the nuclease Bal 31 digestion. After treatment with the Klenow enzyme (PolIK) the plasmid was ligated to the BamHI-linearized plasmid pAZe3ss treated with the Klenow enzyme. DNA ligation was performed as indicated in Materials and Methods. The recombinant plasmids encoding different deletions at the carboxyl end of protein p6 under the control of the λ P_L promoter (pMJc Δ series), contain the oligodeoxynucleotide TGATTGATTGA from plasmid pAZe3ss (20), so that the stop codon TGA is placed downstream of the deleted p6-coding sequences in any possible reading frame. Restriction endonucleases: B, BamHI; Bg, BglI; E, EcoRI; H, HindIII; N, NcoI; P, PstI; S, Sau3AI. CIAP, calf intestinal alkaline phosphatase.

Fig. 2. Analysis of the protein p6 deletion mutants by SDS- polyacrylamide gel electrophoresis. The *E. coli* K12ΔH1Δtrp cells transformed with the different recombinant plasmids were grown at 30°C, shifted to 42°C and 1 h after the shift, samples were either labelled for 2 min with 10 μCi of ³⁵S-methionine, subjected to SDS-gel electrophoresis in 10–20% polyacrylamide gradients and autoradiographed (A), or directly subjected to electrophoresis and stained with Coomassie blue (B). Lanes: a, cells containing pRP8 kept at 30°C as a control; b-h, cells containing the different plasmids, shifted to 42°C; b, pRP8; c, pMJcΔ14; d, pMJcΔ23; e, pMJcΔ38; f, pMJcΔ37; g, pMJcΔ39; h, pMJcΔ44. Arrows indicate the position of the wild-type protein p6.

Protein p6cΔ14 was finally purified by centrifugation for 24 h at 280,000×g in a 15–30% glycerol gradient in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl.

For protein p6 mutants with 23 or more amino acids deleted at the carboxyl end, that became insoluble, a different purification procedure was followed. One g of *E. coli* K-12ΔH1Δtrp cells transformed with the different recombinant plasmids from the pMJcΔ(23–44) series, induced for 0.5–1 h at 42°C, were ground with alumina and extracted with buffer A + 0.3 M NaCl. The lysates were centrifuged for 5 min at 650×g and the supernatants were centrifuged for 15 min at 8,500×g. The pellets were washed 4 times with the same buffer, reextracted with 50 mM Tris-HCl, pH 7.5, 2 M guanidinium-HCl to solubilize the proteins as described (A. Zaballo, J.M. Lázaro, E. Méndez, R.P. Mellado and M. Salas, submitted for publication) and centrifuged for 15 min at 8,500×g. The supernatants were dialyzed against buffer A + 1 M NaCl and then against buffer A. After dialysis, samples were centrifuged for 15 min at 8,500×g and the supernatants were passed through a phosphocellulose column (0.5 ml) equilibrated in buffer A + 50 mM NaCl. After washing with the same buffer, mutant proteins were eluted by addition of buffer A + 0.1–0.2 M NaCl. To concentrate the proteins, the samples were diluted

with buffer A, passed through a second phosphocellulose column (50 μ l) equilibrated in buffer A + 50 mM NaCl and eluted with buffer A + 0.5 M NaCl. As a control the wild-type protein p6 was treated with guanidinium-HCl and dialyzed as described above to compare the activity of the mutant proteins. SDS-polyacrylamide gel electrophoresis was carried out to determine the amount of the mutant proteins at the different purification steps. *In vitro* assay for the formation of protein p3-dAMP initiation complex. DNA replication assays

The *in vitro* assay for the formation of protein p3-dAMP initiation complex was as described (28) except that 20 mM $(\text{NH}_4)_2\text{SO}_4$ was added, and the NaCl concentration was as indicated. A NaCl concentration up to 150 mM can be used without affecting the initiation reaction (13). After 3 min at 30°C the samples were treated with micrococcal nuclease and subjected to SDS-polyacrylamide gel electrophoresis as described (28). Autoradiographs were used for densitometry. The incubation mixture for the DNA replication assay was as described (28), but containing 80 μ M each dGTP, dCTP, dTTP and [α - ^{32}P]dATP (2.5 μ Ci). A NaCl concentration of 100 mM was used to maximize the stimulation by protein p6 in the replication assay (13). After 6 min at 30°C the reaction was stopped by addition of 10 mM EDTA, 0.1 % SDS and the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1 % SDS. Cerenkov radiation was determined in the excluded fractions.

Gel retardation assays

Protein samples were incubated for 30–60 min at 0°C in a final volume of 20 μ l of a buffer containing 12 mM Tris-HCl, pH 7.5, 1 mM EDTA with approximately 1000 cpm (0.2–2 ng) of a ^{32}P -labelled right end ϕ 29 DNA restriction fragment (^{32}P -HindIII L, 269 bp long). Protein-DNA complexes were resolved in low ionic strength non-denaturing polyacrylamide gels, essentially as described (29).

DNase I footprinting

Footprinting was carried out as described (18). The indicated amounts of wild-type protein p6 and the carboxyl end deletion mutants were incubated with approximately 10,000 cpm (2–20 ng) of ^{32}P -labelled HindIII L fragment in a final volume of 20 μ l.

RESULTS

Characterization and purification of the protein p6 mutants

The construction of the recombinant plasmids encoding the protein p6 deletion mutants is shown in Fig. 1. The recombinant plasmids were propagated in the λ lysogen *E. coli* K12 Δ H1 Δ trp where the transcription from the P_L promoter is repressed at 30°C by the thermosensitive cI857 repressor carried by the lysogen host. Normal transcription from the P_L promoter occurs upon heat induction at 42°C (19). Figure 2A shows that expression of the recombinant plasmids gave rise to the synthesis of labelled proteins with the electrophoretic mobilities expected for the wild-type protein p6 (15) (plasmid pRP8, lane b) and for the newly constructed p6 mutants (pMJc Δ series plasmids, lanes c–h). When these cells were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue, overproduced polypeptides with the expected mobilities for the carboxyl deleted p6 mutants were observed (Fig. 2B). The p6 Δ 14 mutant protein was soluble and stable, but when 23 or more amino acids at the carboxyl end of protein p6 were deleted, the proteins became unstable and insoluble (results not shown). The polypeptide p6 Δ 14 was purified as previously described (15). Fig. 3A shows the mutant protein at the final purification step. The polypeptides p6 Δ 23/+IR(p6 Δ 23),

Fig. 3. Analysis by SDS-polyacrylamide gel electrophoresis of the mutant p6 proteins. (A) Purified p6cΔ14 mutant protein (lane a) (glycerol gradient fraction) and purified wild-type protein p6 (lane b). (B) p6cΔ44 mutant protein at various purifications steps: (a) lysate; (b) soluble extract; (c) guanidinium-HCl supernatant; (d) first phosphocellulose, flow-through; (e) first phosphocellulose, 0.2 M NaCl eluate. (C) Purified p6cΔ23 (lane a), p6cΔ38 (lane b), p6cΔ37 (lane c), p6cΔ39 (lane d), p6cΔ44 (lane e) mutant proteins at the final purification step (second phosphocellulose, 0.5 M NaCl eluate) and purified wild-type protein p6 (lane f).

p6cΔ37/+IR(p6cΔ37), p6cΔ38/+DPVID (p6cΔ38), (p6cΔ39/+SGD (p6cΔ39) and p6cΔ44/+GSGD (p6cΔ44) were purified as described in Materials and Methods. Fig. 3B shows the analysis by SDS-polyacrylamide gel electrophoresis of the different fractions of the purification of one of these polypeptides (the p6cΔ44 mutant protein). Fig. 3C shows the different mutant proteins at the final purification step.

Activity of the p6 mutant proteins in the formation of the initiation complex and ϕ 29 DNA replication

Highly purified protein p6 deletion mutants were tested for their stimulation in the formation of the p3-dAMP initiation complex as indicated in Materials and Methods. Fig. 4A shows that the p6cΔ14 deletion mutant strongly stimulated the formation of the p3-dAMP initiation complex, even to a higher extent than the wild-type protein p6. Figure 4B shows that the p6cΔ23 deletion mutant stimulated the formation of the p3-dAMP complex, although the activity was reduced to about 28% of that of the wild-type protein (see also Fig. 7). The stimulation of the initiation reaction by protein p6 decreased considerably when 37 to 39 amino acids were deleted and was not detected when 44 amino acids were removed (see also Fig. 7).

Figure 5A shows that the p6cΔ14 mutant protein increased the amount of ϕ 29 DNA synthesized being the stimulation about 2-fold higher than that obtained with the wild-type protein p6. Figure 5B shows that larger deletions at the carboxyl end of protein p6 decreased

Fig. 4. Effect of the p6 mutant proteins on the *in vitro* synthesis of the p3-dAMP initiation complex. (A) p3-dAMP complex formation at 48 mM NaCl in the presence of purified proteins p2 (18 ng) and p3 (15 ng) and ϕ 29 DNA-protein p3 (500 ng) as template in the absence or presence of the indicated amounts of purified wild-type protein p6, or p6cΔ14 deletion mutant protein. (B) p3-dAMP complex formed at 100 mM NaCl in the presence of purified protein p2 (18 ng) and p3 (15 ng) and ϕ 29 DNA-protein p3 (500 ng) in the absence or presence of the indicated amounts of purified wild-type protein p6, or deletion mutant proteins. Quantitation of the labelled p3-dAMP band was done by densitometry of the autoradiographs (see Fig. 7 for the comparative values).

the stimulatory effect on ϕ 29 DNA replication with respect to the wild-type protein. Thus, the stimulation by the p6cΔ23 mutant protein was about 5-fold smaller than that by the wild-type protein p6. The activity of mutants p6cΔ37, p6cΔ38 and p6cΔ39 was less than 10% of that of the wild-type protein and no stimulation was detected when mutant p6cΔ44, with 44 amino acids deleted at the carboxyl end, was used (see Fig. 7). The stimulation of ϕ 29 DNA replication by the different mutant proteins correlated well with the data obtained on the formation of the initiation complex.

Fig. 5. Effect of the p6 mutant proteins on the *in vitro* replication of ϕ 29 DNA-protein p3. (A) The replication assay was carried out at 100 mM NaCl at 30°C for the indicated times in the absence or presence of 2 μ g of wild-type protein p6 or p6cΔ14 deletion mutant. (B) The replication assay was carried out at 100 mM NaCl at 30°C for 6 min in the absence or presence of the indicated amounts of wild-type protein p6 or deletion mutants.

Fig. 6. DNase I footprints of wild-type protein p6 and carboxyl end deletion mutants to the right end ϕ 29 DNA fragment HindIII L. (A) DNase I footprint in the absence or presence of the indicated amounts of wild-type protein p6 or the p6cΔ14 deletion mutant. Products of a purine-specific chemical chain degradation reaction were run in parallel (M). (B) DNase I footprint in the absence or presence of the indicated amounts of wild-type protein p6 or p6cΔ23, p6cΔ38, p6cΔ37, p6cΔ39 or p6cΔ44 deletion mutants.

Binding of the mutant proteins to double-stranded ϕ 29 DNA

The mutant proteins were tested for the formation of protein-DNA complexes by gel retardation assays and by DNase I footprinting. All proteins produced DNA retardation, although the amount of the p6cΔ23–44 mutant proteins required was about 2–3 fold higher than that of the wild-type protein p6 or the protein p6cΔ14 mutant (results not shown).

Figure 6A shows that the p6cΔ14 mutant protein was able to bind to the HindIII L restriction fragment from the right ϕ 29 DNA end producing a characteristic pattern of hypersensitive bands spaced about 24 nucleotides apart along most of the fragment, flanking protected regions, similarly to the pattern obtained with the wild-type protein p6 (18).

Fig. 7. Summary of activity of protein p6 carboxy-terminal deletion mutants in the stimulation of replication and specific interaction with the ϕ29 DNA ends. The inferred amino acid sequences at the carboxyl end of the wild-type protein p6 and the deleted p6 proteins are shown. The arrows mark the extent of each deletion. Fusion between vector and gene 6 sequences generates the indicated amino acid substitutions. The values corresponding to the stimulation of initiation and elongation by the wild-type protein p6 and deletion mutants (in %) were taken from Figs. 4 and 5 by subtraction of the corresponding values in the absence of these proteins. The amount (μg) of protein p6 required for DNA binding as determined by DNase I footprinting, were taken from Fig. 6.

About half the amount of the p6cΔ14 deletion mutant than of the wild-type protein p6 was required to produce this characteristic digestion pattern. Figure 6B shows that the same pattern of digested bands was obtained in the presence of the p6cΔ23, p6cΔ37 and p6cΔ38 mutant proteins, although at least 10 μg of the p6cΔ23 and p6cΔ38 and 12 μg of the p6cΔ37 mutant proteins were required. The pattern of digested bands obtained in the presence of the p6cΔ39 and p6cΔ44 mutant proteins was essentially the same as that obtained in the absence of protein p6. Higher amounts of protein could not be used because of the resulting high salt concentration that inhibits the specific binding of protein p6 to the terminal ϕ29 DNA fragment. The fact that the p6cΔ39 and p6cΔ44 mutant proteins produced DNA retardation could reflect an unspecific binding of the mutant proteins to the DNA.

Figure 7 summarizes the results obtained with the different protein p6 mutants relating its activity in ϕ29 DNA replication with its binding to the ϕ29 DNA ends.

DISCUSSION

To map the location of functional domains in protein p6 we have constructed deletion mutants at the carboxyl end of the protein and studied their activity in the initiation of ϕ29 DNA replication and their capacity to interact with the ϕ29 DNA ends. A 14 amino acids deletion, which removes 6 out of the 10 negative charges present within the 20 carboxy-terminal amino acids, slightly increased the activity of protein p6 in ϕ29 DNA replication. In addition, the specific interaction of this deleted protein with the ϕ29 DNA right end was observed at a concentration half the one required by the wild-type protein p6, possibly reflecting the reduction of negative charges in the protein that could decrease the repulsion between the DNA and the protein. This improvement in DNA binding is likely to be responsible of the p6cΔ14 overactivity. The suggestion that the acidic region of protein p6 is dispensable is also supported by the sequence of the corresponding protein of the ϕ29-related bacteriophage PZA, which is identical to the ϕ29 protein p6 sequence except for an internal deletion from amino acid residues 89 to 96 (30), that corresponds

to the major part of this acidic region. Deletions of 23, 37, 38 and 39 amino acids further inside the protein progressively diminished the activity in the initiation of ϕ 29 DNA replication and the affinity of the proteins for the ϕ 29 DNA right end. The mutant protein lacking the 44 carboxyl-end amino acids was completely inactive. The low activity of the p6 mutant proteins with deletions from 23 to 39 amino acids at the carboxyl end was partially compensated by increasing the amount of the protein. These results could be explained if this region of protein p6 is involved in protein-protein interaction for the formation of dimers (15) which are likely to be the active form of the protein. Alternatively, this region could be needed to maintain an active protein p6 conformation. This functional domain was probably lost when 44 amino acids at the carboxyl end of protein p6 were removed.

Protein p6, when assayed at salt conditions close to the *in vivo* ones (~ 100 mM), has been shown to play an important role in the coupling of the initiation and elongation steps in ϕ 29 DNA replication (13). In that respect, there is a good correlation between the results on the stimulation of replication induced by the mutant proteins with those obtained in the formation of the p3-dAMP initiation complex. By deletion analysis of ϕ 29 DNA terminal sequences we have shown the existence of a good correlation between binding of protein p6 and stimulation of replication (16), suggesting that the formation of a protein p6-DNA nucleoprotein complex may be the structural basis for the p6 activity in the initiation of replication. In agreement with this, the results obtained with the series of carboxy-terminal mutants of protein p6 presented here indicate that the stimulation of ϕ 29 DNA replication by protein p6 depends on its specific interaction with the ϕ 29 DNA ends.

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