Initiation of phage ϕ29 DNA replication by the terminal protein modified at the carboxyl end

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ABSTRACT

A mutant at the carboxyl end of the terminal protein, p3, of phage Ø29 DNA has been constructed by inserting an oligonucleotide containing the stop translation codon TGA in the three possible reading frames, immediately downstream of a Ø29 DNA fragment coding for all but the last five amino acids of protein p3. The activity in the formation of the p3-dAMP initiation complex in vitro of this mutant as well as another one previously isolated, also mutated at the carboxyl end, have been tested. The results obtained suggest that an intact carboxyl end in the Ø29 terminal protein is essential for its normal primer function in DNA replication.

INTRODUCTION

The Bacillus subtilis phage Ø29 has a linear, double-stranded DNA 18,000 base pairs long (1) with a viral protein, p3, covalently bound to the two 5' ends (2-5). Ø29 DNA replication starts at either end of the DNA molecule and proceeds by strand displacement (6-8). Protein p3 is the product of gene 3, shown to be involved in the initiation of the viral DNA replication in vivo (9). The terminal protein, p3, primes the initiation of Ø29 DNA replication in vitro, by formation of a p3-dAMP covalent complex that provides the 3' OH group needed for elongation. The reaction also requires Ø29 DNA-protein p3 as template (10,11) and the presence of the product of gene 2 (12), which has been previously shown to be also involved in the initiation of Ø29 DNA replication in vivo (9).

The region of the viral genome containing gene 3 has been placed under the control of the bacteriophage λ thermoinducible P_L promoter contained in the plasmid pPLc28 (13) allowing in this way the overproduction of a protein p3 modified at its carboxyl

end, p3', with the last four amino acids of the normal protein replaced by eight new ones (14). We report in this paper the construction and overproduction in a similar manner of a new p3 mutant at the carboxyl end, p3", having the length of the normal polypeptide but the last three amino acids changed. We have used both mutant proteins, p3' and p3", to study the initiation of Ø29 DNA replication *in vitro*, the activity being 15% and 12%, respectively, from that found with the normal protein p3. These results suggest the requirement of a non-altered carboxyl end for the protein p3 to be fully functional.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *E. coli* strain HB101 (15) was obtained from N.E. Murray. *E. coli* K12ΔH1Δtrp (16) has been described elsewhere (13). Plasmid pKTH601 (17) was obtained from R.F. Pettersson and used as a cloning vector as well as plasmid pPLc28 (13) which was obtained from E. Remaut. Plasmids pKC30 A1 and pKC30 B1 (18) were obtained from J.A. García. Plasmid pRMw51 has been described before (14).

Enzymes

Restriction enzymes were from New England Biolabs and used according to the supplier. T4 DNA ligase was purified by J.M. Lázaro from *E. coli* cells harbouring the plasmid pPLc28 ligase 8 obtained from E. Remaut. Fungal proteinase K was from Merck and micrococcal nuclease from Worthington.

DNA preparations

The Ø29 DNA-protein p3 complex (10) and proteinase K-treated Ø29 DNA (19) were obtained as described. Extracts from *B. subtilis* infected with the Ø29 mutant *sus*3(91) (20) were obtained from J.A. García. Plasmid DNA was prepared according to Clewell (21). The Ø29 DNA Hind III G fragment purification, ligation, transformation, antibiotic selection of recombinants, DNA minipreparations and agarose gel electrophoresis were as described (14).

Characterization of the protein p3 mutants

The polypeptides synthesized upon induction of *E. coli* K12ΔH1Δtrp harbouring the recombinant plasmids were radioactively labelled with ^{35}S-methionine and analysed by 20% polyacryl-

amide electrophoresis in the presence of SDS followed by autoradiography (14). The relative production of the mutant proteins was determined by densitometry of the autoradiographs and expressed as percentage of the total labelled protein in the gel lane (14).

E. coli cultures transformed with the recombinant plasmids were induced and lysed as described (18). The presence of the mutant polypeptides in the extracts was tested by a binding radioimmunoassay (22) modified as it will be described elsewhere (23). Basically the protein in the extracts was bound to the wells of a polystyrene plate and then incubated with rabbit anti-p3 serum (a gift from I. Prieto) followed, after washing, by incubation with {^{125}I}-protein A. The radioactivity bound was removed with 2 M NaOH and counted.

Assay for the in vitro formation of protein p3-dAMP initiation complex

Cell cultures carrying the recombinant plasmids were grown in L broth, induced at 42°C for 3 hr, and the cells were collected, washed, ground with twice their weight of alumina, centrifuged at low-speed to remove bacterial debris, and passed through a Sephadex G-50 column to eliminate endogenous nucleotides, as described (18). The incubation mixture for the initiation reaction was as described by Peñalva and Salas (10) and contained, in a final volume of 0.05 ml, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 0.25 µM {α-^{32}P}dATP (5 µCi), 35 µg of protein from extracts from sus3 infected B. subtilis prepared as described (11), 5-45 µg of protein from extracts of E. coli harbouring the recombinant plasmids as source of protein p3, prepared as described (18) and about 0.6 µg of Ø29 DNA-protein p3 as template. After 20 min at 30°C the samples were processed and the p3-dAMP complexes formed analyzed by 10% polyacrylamide gel electrophoresis in the presence of SDS as described (10). The gels were dried and autoradiographed with intensifying screens at -70°C. The amount of p3-dAMP complex formed was quantitated by densitometry of the autoradiographs. Several doses of the different sources of protein p3 were assayed to obtain a linear response in the amount of p3-dAMP formed.

RESULTS AND DISCUSSION

Construction of recombinant plasmids

The fragment Hind III G of Ø29 DNA contains the information for the synthesis of protein p3 except for the last five amino acids at the carboxyl end (24,25). The plasmid pKTH601 (17) contains the synthetic oligonucleotide TGATTGATTGA flanked by Hind III and Bam HI targets replacing the natural Hind III-Bam HI fragment of pBR322 so that any sequence inserted at the Hind III site of pKTH601 will be followed downstream by the translation stop codon TGA in any of the three possible reading frames. Thus, the cloning of the Ø29 DNA Hind III G fragment in pKTH601 will ensure that translation termination occurs shortly after the inserted viral sequences. In fact, a mutant protein p3 (p3") could be synthesized that would have the same length as the normal protein p3 but containing three different amino acids at the carboxyl end. The construction of such a recombinant (pRMt12) is shown in Figure 1.

The Eco RI-Bam HI fragment from pRMt12,containing the Ø29 DNA sequences cloned in the appropriate orientation (Fig. 1),was transferred to the plasmid pPLc28 (13) previously cut with Eco RI and Bam HI together, so that the viral sequences were under the direct control of the λ P_L promoter. The plasmid pRMt12 was also cut with Pst I to avoid the cloning of any other fragment but the desired one. The recombinant plasmid pRMt121, obtained in this manner is shown in Figure 1.

The plasmid pRMw51 has been described before (14) and was constructed in a similar way as pRMt121, but pBR322 (26) was used instead pKTH601, so that the protein p3 encoded in pRMw51, named p3', is 4 amino acids longer than the normal one and differs from it in a total of eight amino acids at the carboxyl end of the molecule (14).

Plasmids pKC30 A1 and pKC30 B1 (18) contain the Ø29 DNA sequences coding for a normal protein p3 under the control of the phage λ P_L promoter harboured in the plasmid pKC30 (27). The plasmid pKC30 A1 carries the viral DNA sequences inserted in the correct orientation, whereas pKC30 B1 carries them in the opposite one, so that upon induction of the P_L promoter a normal protein p3 is synthesized only in the cell cultures containing

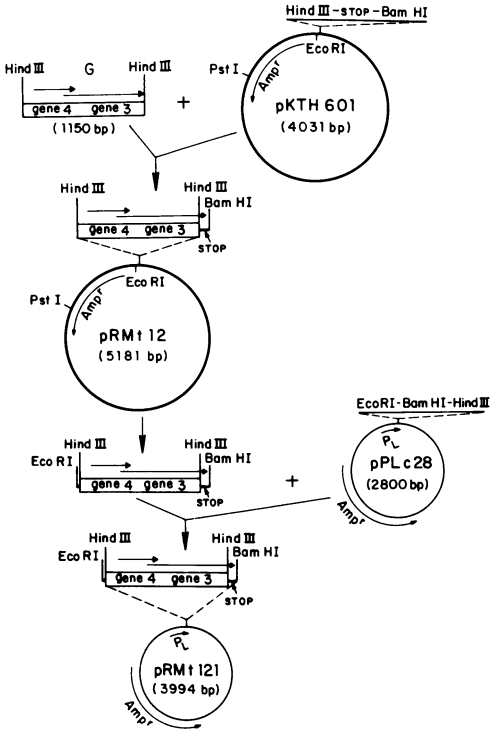

Fig.1. Construction of recombinant plasmid pRMt121. The arrows above genes 3 and 4 indicate their extension and direction of transcription in Ø29 DNA with respect to the P_L promoter in pRMt121. The direction of transcription of the ampicillin resistance gene is also indicated.

the plasmid pKC30 A1 (18). Both the pKC30 and the pPLc28 derivative plasmids were propagated in the lysogen E. coli K12ΔH1Δtrp so that the transcription from the P_L promoter will be repressed at 30°C by the thermosensitive cI857 repressor carried by the lysogen host, being the normal transcription from P_L fully restored upon heat induction (13).

Inducible synthesis of the Ø29 proteins coded by pRMt121

Bacteria carrying the plasmid pRMt121 were grown as described (14). After 1 hr at 30°C one half of the culture was shifted to 42°C and at 90 and 180 min after induction, samples from the 42°C culture were labelled with ^{35}S-methionine for 10 min. As a control, samples from the cultures kept at 30°C were equally labelled. Expression of the recombinant plasmid produced, in addi-

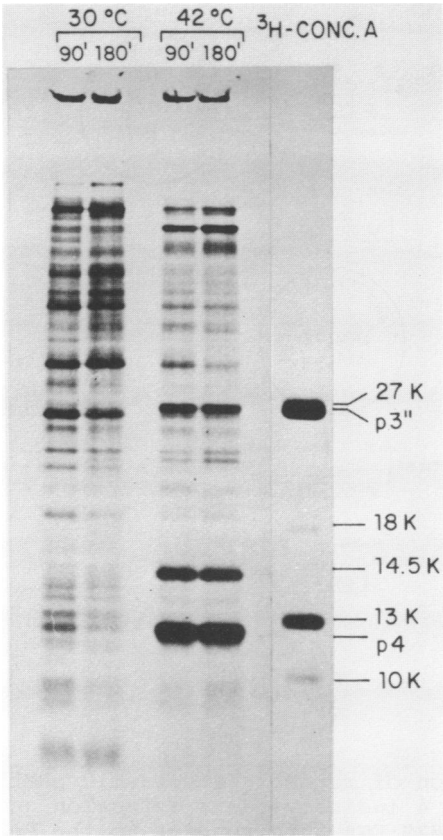

Fig.2. Induced synthesis of protein p3" directed by plasmid pRMt121. Bacterial cultures harbouring the plasmid pRMt121 were grown, labelled with ^{35}S-methionine and subjected to SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The starting time of each labelling period (in min) and the temperature of incubation are indicated above the lanes. ^{3}H-concanavalin A peptides (The Radiochemical Centre, Amersham) of known molecular weights (29) were used as markers. The molecular weight of the markers in K daltons is given at the right, as well as the position of proteins p3", p4 and a 14.5 K induced polypeptide.

tion to protein p4 (14), a protein with the electrophoretic mobility expected for the newly constructed protein p3" (Fig. 2). Both proteins were missing in the cultures kept at 30^{o}C. A new polypeptide with a molecular weight of 14,500 was also labelled in the induced cultures and corresponds to the fusion of pKTH601 sequences contained in the cloned Eco RI-Bam HI fragment with

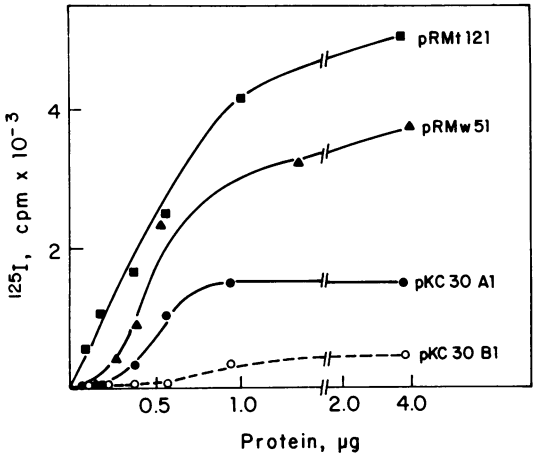

Fig.3. Radioimmunoassay of protein p3 in extracts from cells carrying the recombinant plasmids. The assay was carried out as described in Materials and Methods by addition of increasing amounts of the extracts to a polystyrene multiwell plate followed by incubation, first with anti-p3 serum and then with {125I}-protein A. The radioactivity bound to each well was counted.

those of plasmid pPLc28 (13,17,28). The level of the mutant protein p3" synthesized under these conditions reached about 9% of de novo made protein at both 90 and 180 min after induction. The electrophoretic mobility of protein p3" coincides with that of normal protein p3 (results not shown).

Characterization of the p3 mutants

To asses that the extracts prepared from cells transformed by the recombinant plasmids contained the p3 mutants, a radioimmunoassay with anti p3-serum was carried out. Fig. 3 shows that {^{125}I}-protein A was specifically bound in the case of cell extracts containing either plasmid pRMw51, which directs the synthesis of protein p3', or plasmid pRMt121 which codes for protein p3". {^{125}I}-protein A was also bound when cell extracts carrying the plasmid pKC30 A1, which produces a normal p3, were used whereas in the case of the plasmid pKC30 B1, which does not produce p3, very little binding of {^{125}I}-protein A was detected.

The difference in the amount of radioactivity bound in each case (see Table 1) correlates reasonably well with the difference in the level of synthesis upon induction of the normal and

Table 1. Effect of the mutations of protein p3 on the formation of the initiation complex

Plasmid	protein	activity,%[a]	ratio[b]	corrected activity, %[c]
pKC30 A1	p3	100	1.0	100
pRMw51	p3'	32	2.1	15
pRMt121	p3"	45	3.7	12

Extracts from E. coli harbouring the different recombinant plasmids were prepared as described in Materials and Methods. The activity of the normal and the mutated p3 proteins in the formation of the p3-dAMP initiation complex was tested in the in vitro system.

[a]Percentage of the activity (referred to total protein) in the formation of p3-dAMP as determined by densitometry of the autoradiographs. The values given are average from 2 experiments done with different sets of extracts.

[b]Ratio of relative p3 present in the extracts as determined by radioimmunoassay. Values are average from 2 experiments done with different sets of extracts.

[c]Activity corrected for the relative amount of p3 protein present in the different extracts.

mutated p3 proteins. Normal protein p3 is produced in approximately 3% of the total de novo protein made (results not shown,18) and proteins p3' and p3" account for about 6% (14) and 9%, respectively. Therefore, the amount of synthesis of protein p3' and p3" is roughly 2 and 3 times more than that of protein p3.

Activity of the protein p3 mutants in the formation of the initiation complex p3-dAMP

Extracts from E. coli transformed with the recombinant plasmids pKC30 A1, pRMw51 and pRMt121, which produce the normal protein p3 and the mutant proteins p3' and p3", respectively, were tested for the formation in vitro of the p3-dAMP complex in a complementation assay using extracts from B. subtilis infected with a Ø29 sus mutant in gene 3. Figure 4 shows that in the presence of Ø29 DNA-protein p3 as template, the addition of any of the three extracts results in the labelling of the corresponding protein p3 with {α-^{32}P}dATP, that is in the formation of the p3-dAMP initiation complex. The reaction was dependent on Ø29 DNA-protein p3 since proteinase K-treated Ø29 DNA did not act as

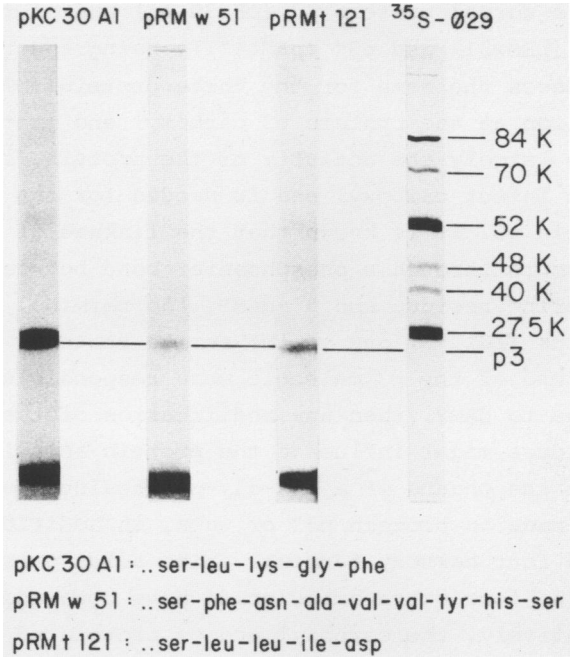

Fig. 4. Synthesis of the p3-dAMP complex in vitro. Extracts from E. coli K12ΔH1Δtrp containing the recombinant plasmids indicated above the lanes were prepared as described in Materials and Methods. E. coli extracts from cells containing the recombinant plasmids (~11µg) were added to the incubation mixture as indicated in Materials and Methods. The p3-dAMP complexes formed were analyzed by polyacrylamide gel electrophoresis. The lower part of the figure shows the predicted amino acid sequence at the carboxyl end of the normal p3 (pKC30 A1) and mutated p3' (pRMw51) and p3" (pRMt121) polypeptides.

template (results not shown). Densitometry of the ^{32}P-labelled p3 in each case showed that the amount of p3-dAMP complex formed by the extracts containing plasmids pRMw51 and pRMt121 was approximately 32% and 45% of that formed by the extracts containing the plasmid pKC30 A1 (Table 1). These values have to be corrected by the relative level of production of the different p3 polypeptides as estimated by the radioimmunoassay with anti-p3 serum. Table 1 shows that when this correction is made, the activity of the mutant proteins p3' and p3" goes down to about 15% and 12%, respectively, from that of the normal protein p3. The lower part of Figure 4 shows the predicted amino acid sequence at the carbo-

xyl end of the normal protein p3 (pKC30 A1) and of the mutant proteins p3' (pRMw51) and p3" (pRMt121), being the rest of the peptide sequences the same for the three proteins. The fact that the modification at the protein p3 carboxyl end in the two mutants affects largely the activity of the protein, strongly suggests that an intact carboxyl end is needed for the full function of protein p3. It is known that the linkage of protein p3 to Ø29 DNA occurs through a phosphoester bond between the OH group of a serine residue and 5'-dAMP, the terminal nucleotide at both 5' ends (30). If any of the serine resudues present at the carboxyl end of the p3 molecule were responsible for the covalent linkage to dAMP, then any modification of the region next to these residues might influence the protein activity, as it is the case when the change of a lys-gly-phe residue for a leu-ile-asp has been made in protein p3" or when, in addition to the change of the four carboxy terminal amino acids present in the normal protein, four extra amino acids have been added (protein p3'). Alternatively, the carboxyl end of protein p3 could be either forming part of an active center in the molecule or being absolutely required to built up an stable conformation of the protein.

A way to produce and select for short deletion mutants at the carboxyl end of protein p3 is currently being developed to determine how essential is the carboxyl end for the function of protein p3 in the initiation of Ø29 DNA replication.

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