Factors involved in the initiation of phage ϕ 29 DNA replication *in vitro*: requirement of the gene 2 product for the formation of the protein p3-dAMP complex

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ABSTRACT

To study the requirements for the *in vitro* formation of the protein p3-dAMP complex, the first step in ϕ 29 DNA replication, extracts from *B. subtilis* infected with ϕ 29 mutants in genes 2, 3, 5, 6 and 17, involved in DNA synthesis, have been used. The formation of the initiation complex is completely dependent on the presence of a functional gene 2 product, in addition to protein p3 and ϕ 29 DNA-protein p3 as template. ATP is also required, although it can be replaced by other nucleotides. The products of genes 5, 6 and 17 do not seem to be needed in the formation of the initiation complex.

Inhibitors of the host DNA polymerase III, DNA gyrase or RNA polymerase had no effect on the formation of the protein p3-dAMP complex, suggesting that these proteins are not involved in the initiation of ϕ 29 DNA replication. ddATP or aphidicolin, inhibitors of DNA chain elongation, had also no effect on the formation of the initiation complex.

INTRODUCTION

Bacillus subtilis phage ϕ 29 contains a linear, double-stranded DNA of about 18 000 base pairs (1) with a protein covalently bound to the two 5' ends (2-5). This protein is the product of gene 3, p3 (2) and is linked to the DNA by a phosphoester bond between the OH group of a serine residue and 5'-dAMP, the terminal nucleotide at both 5' ends (6). The nucleotide sequence of the DNA region shown to contain gene 3 has been determined and a molecular weight of 31 049 can be deduced for protein p3 (7,8). Genes 2, 3, 5, 6 and 17 are involved in ϕ 29 DNA replication (9-12). Shift-up experiments using *ts* mutants showed that genes 2 and 3 are involved in an initiation step, and genes 5 and 6 in elongation or maturation steps (12).

Phage ϕ 29 DNA replication starts at either DNA end, non simultaneously, and proceeds by a mechanism of strand-displacement

(13-15). Adenovirus DNA, which also has a protein covalently linked to the 5' ends (16,17), replicates by a similar strand-displacement mechanism (18,19).

An important problem in the replication of a linear DNA such as that of phage ϕ 29 and adenovirus, is how the DNA chain is initiated at the 5' ends. In the model proposed for the initiation of ϕ 29 (12-14) and adenovirus DNA replication (16) a free molecule of the terminal protein primes replication by reaction with the dNTP at the 5' ends and formation of a protein-dNMP covalent complex that provides the 3'OH group needed for elongation by the DNA polymerase. This mechanism is supported by the finding of the formation in vitro of a covalent complex between protein p3 and dAMP (20,21) and between the precursor of the adenovirus terminal protein and dCMP, the nucleotide at the 5' ends of adenovirus DNA (22-26).

With the use of extracts from B. subtilis infected with ϕ 29 mutants in genes 2, 3, 5, 6 and 17 we show in this paper that the in vitro formation of the protein p3-dAMP initiation complex requires, besides protein p3, the product of gene 2. The products of genes 5, 6 or 17 do not seem to be involved in vitro in the initiation step. The results obtained using drugs which inhibit host enzymes needed for bacterial DNA replication suggest that host DNA polymerase III, DNA gyrase and RNA polymerase are not required in the formation in vitro of the p3-dAMP initiation complex.

MATERIALS AND METHODS

Bacterial strains and phage

B. subtilis F25-1, lacking DNA polymerase I activity (27), obtained from N. Cozzarelli and B. subtilis 110NA $\text{try}^- \text{spoA}^-$ (28), were used as non-permissive hosts. B. subtilis MO-101-P $\text{spoA}^- \text{thr}^- \{\text{met}^-\}^+ \text{su}^{+44}$ (29), was used as permissive host.

The suppressor-sensitive (sus) mutants of phage ϕ 29, sus2 (513), sus2 (515), sus3 (91), sus6 (626) and sus17 (112) were from the collection of Moreno et al. (28), and sus17 (741) from that of Reilly et al. (30). The temperature-sensitive (ts) mutants ts2 (98) and ts5 (17) were from the collection of Talavera et al. (31).

Materials

{ α -³²P}dATP (410 Ci/mmol) was obtained from The Radiochemical Centre, Amersham. Micrococcal nuclease was from Worthington. Nalidixic acid, novobiocine, rifampicin, chloramphenicol, nucleoside 5'-triphosphates, deoxynucleoside 5'-triphosphates and β , γ -methyleneadenosine 5'-triphosphate (AMP-PCP) were from Sigma Chemical Co. and β , γ -imidoadenosine 5'-triphosphate (AMP-PNP) and 2',3'-dideoxyadenosine-5'-triphosphate (ddATP) were from P-L Biochemicals, Inc. Aphidicolin and 6-(p-hydroxyphenylazo)uracil were a gift from Imperial Chemical Industries. ϕ 29 DNA-protein p3 complex was prepared as described (20).

Growth of cells and preparation of extracts

B. subtilis F25-1 su⁻, lacking DNA polymerase I activity, was grown at 37°C and infected with wild-type phage ϕ 29 or with different sus mutants as described (20). In the case of ts mutants, parallel infections were carried out at 30°C and 44°C. The cells were harvested at either 45 min postinfection (experiments at 37°C or 44°C) or 55 min postinfection (experiments at 30°C).

Lysates were prepared from frozen cells as described (20). After centrifugation for 30 min at 40 000 x g, the supernatant was precipitated with ammonium sulfate to 70% saturation and dialyzed (S40-fraction). The pellet was extracted by phase partition with polyethyleneglycol 8000-dextrane 500 (PEG-fraction) as described (20). When indicated, the supernatant was passed, prior to the ammonium sulfate precipitation, through a DEAE-cellulose column, to remove nucleic acids (DEAE-fraction) (20).

Assay for formation of protein p3-dAMP covalent complex (initiation reaction)

The standard incubation mixture for the initiation reaction contained, in a final volume of 0.05 ml, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM ATP, 0.25 μ M { α -³²P}dATP (5 μ Ci) and about 75 μ g of protein from the different fractions. When indicated, 0.5 μ g of ϕ 29 DNA-protein p3 complex was added as template. After incubation for 20 min at 30°C the samples were processed as described (20), including the treatment with micrococcal nuclease. The samples were subjected to electrophoresis in polyacrylamide slab gels containing 10% acrylamide and 0.1% SDS (32). The gels

were dried and autoradiographed with intensifying screens at -70°C .

RESULTS

Requirement of the products of genes 2 and 3 for the in vitro formation of the p3-dAMP initiation complex

When the DEAE-fraction from B. subtilis infected with mutant sus3(91) was incubated in vitro with $\{\alpha\text{-}^{32}\text{P}\}$ dATP and $\phi 29$ DNA-protein p3 as described in Materials and Methods, no formation of a ^{32}P -labelled band corresponding to the p3-dAMP initiation complex was seen in the gels (Fig. 1A and B, lane a; see also lane g in Figs. 2, 3 and 4). This is the expected result since no intact protein p3 should be synthesized in the sus3-infected cells. Neither the S40- nor the PEG-fraction, prepared from B. subtilis infected with mutant sus2(513), both containing protein p3 (20), gave rise to the formation of the ^{32}P -labelled p3-dAMP band in the initiation reaction (Fig. 1A, lanes b and c). However, they were complemented with the DEAE-fraction from sus3-infected cells (Fig. 1A, lanes d and e). Mixing the S40- and PEG-fractions from the sus2-infected extracts did not restore the ability to form the p3-dAMP initiation complex (results not shown). To determine whether the results obtained with mutant sus2(513) were specific of this particular mutant, due to some cryptic mutation, or other mutants in gene 2 behaved in a similar way, the PEG-fraction was prepared from B. subtilis infected with mutant sus2(515) and tested in the formation of the p3-dAMP complex. As shown in Fig. 1B (lanes b-d), this fraction was only active when complemented with the DEAE-fraction from sus3-infected cells and it required the addition of $\phi 29$ DNA-protein p3 as template. Moreover, the S40-fraction prepared from cells infected at 44°C with mutant ts2(98) did not give rise to the formation of the initiation complex unless supplemented with the DEAE-fraction from the sus3-infected cells and $\phi 29$ DNA-protein p3 as template (Fig. 2, lanes d-f). When the infection with mutant ts2(98) was done at 30°C initiation complex was formed (Fig. 2, lanes a-c). All the above results show that the gene 2 product is required, in addition to protein p3, in the formation of the protein p3-dAMP initiation complex.

Fig. 1. Effect of *sus* mutations in genes 2 or 3 on the *in vitro* formation of the protein p3-dAMP initiation complex. **A.** Fractions (S40 or PEG) from *B. subtilis* infected with mutant *sus2*(513) or DEAE-fraction from *B. subtilis* infected with mutant *sus3*(91) were incubated with [α - 32 P]dATP, ATP and ϕ 29 DNA-protein p3 (except where indicated) as described in Materials and Methods. After treatment with micrococcal nuclease the samples were subjected to SDS-polyacrylamide gel electrophoresis. Lanes : a. *sus3*(91); b. *sus2*(513), S40-fraction. c. *sus2*(513), PEG-fraction. d. *sus2*(513), S40-fraction + *sus3*(91). e. *sus2*(513), PEG-fraction + *sus3*(91). f. Markers of known molecular weight (the figures indicate K daltons). **B.** PEG-fraction from cells infected with mutant *sus2*(515) or DEAE-fraction from cells infected with mutant *sus3*(91) were incubated as in A. Lanes : a. *sus3*(91); b. *sus2*(515); c. *sus2*(515) + *sus3*(91); d. as c without ϕ 29 DNA-protein p3; e. Markers of known molecular weight (the figures indicate K daltons).

Effect of mutations in genes 5, 6 and 17 on the formation of the initiation complex

Using available conditional-lethal mutants of phage ϕ 29 we tried to answer the question if any other known genes, needed *in vivo* for DNA replication, are involved in the formation of the initiation complex, *in vitro*. Fig. 3 shows that the PEG-fraction prepared from cells infected at 30°C or 44°C with mutant *ts5*(17) was active in the formation of the 32 P-labelled p3-dAMP complex

Fig. 2. Effect of a *ts* mutation in gene 2 on the *in vitro* formation of the protein p3-dAMP initiation complex. S40-fraction prepared from *B. subtilis* infected at 30°C or 44°C with mutant *ts2(98)* was incubated for 20 min at 30°C with $\{\alpha\text{-}^{32}\text{P}\}$ dATP, ATP and ϕ 29 DNA-protein p3 (except where indicated) as described in Materials and Methods. When indicated, DEAE-fraction from *B. subtilis* infected with mutant *sus3(91)* was added. After treatment with micrococcal nuclease the samples were subjected to SDS-polyacrylamide gel electrophoresis. a. *ts2(98)* - 30°C; b. *ts2(98)* - 30°C + *sus3(91)*; c. as b without ϕ 29 DNA-protein p3; d. *ts2(98)* - 44°C; e. *ts2(98)* - 44°C + *sus3(91)*; f. as e without ϕ 29 DNA-protein p3; g. *sus3(91)*; h. Markers of known molecular weight (the figures indicate K daltons).

when supplemented with ϕ 29 DNA-protein p3 as a template (Fig. 3, lanes a, b, d and e). The reaction was stimulated when the DEAE-fraction from *sus3*-infected cells was added (Fig. 3, lanes c and f).

Figure 4 shows that the PEG-fraction prepared from cells infected with mutants *sus6(626)* or *sus17(741)* is active in the formation of the initiation complex when ϕ 29 DNA-protein p3 is added as a template (lanes a and d). No reaction takes place in the absence of the template (lanes b and e) and no stimulation is observed when, in addition, the DEAE-fraction from *sus3*-infected cells is added (lanes c and f). Similar results to those reported for mutant *sus17(741)* were obtained when other mutant in gene 17, *sus17(112)*, was used (not shown).

Fig. 3. Effect of a *ts* mutation in gene 5 on the *in vitro* formation of the protein p3-dAMP initiation complex. PEG-fraction prepared from *B. subtilis* infected at 30°C or 44°C with mutant *ts*5 (17) was incubated for 20 min at 30°C with [α -³²P]dATP, ATP and ϕ 29 DNA-protein p3 (except where indicated) as described in Materials and Methods. When indicated, DEAE-fraction from *B. subtilis* infected with mutant *sus*3(91) was added. After treatment with micrococcal nuclease the samples were subjected to SDS-polyacrylamide gel electrophoresis. a. *ts*5(17) - 30°C; b. as a without ϕ 29 DNA-protein p3; c. *ts*5(17) - 30°C + *sus*3(91); d. *ts*5(17) - 44°C; e. as d without ϕ 29 DNA-protein p3; f. *ts*5 - 44°C + *sus*3(91); g. *sus*3(91); h. Markers of known molecular weight (the figures indicate K daltons).

Effect of inhibitors of replication, transcription and translation on the formation of the initiation complex

As an approach to search for host functions needed for the initiation step, different inhibitors of DNA replication were tested for their effect on the formation of the initiation complex. Table 1 shows that neither nalidixic acid nor novobiocine, inhibitors of the A and B subunits of the DNA gyrase, respectively (33), affected the formation of the p3-dAMP complex. Aphidicolin, which has been reported to inhibit ϕ 29 DNA synthesis *in vivo* (34) and 6-(p-hydroxyphenylazo)uracil, an inhibitor of

Fig. 4. Effect of a *sus* mutation in genes 6 or 17 on the *in vitro* formation of the protein p3-dAMP initiation complex. PEG-fraction prepared from *B. subtilis* infected with mutants *sus*6 (626) or *sus*17(741) was incubated for 20 min at 30°C with [α - 32 P] dATP, ATP and ϕ 29 DNA-protein p3 (except where indicated) as described in Materials and Methods. When indicated, DEAE-fraction from *B. subtilis* infected with mutant *sus*3(91) was added. After treatment with micrococcal nuclease the samples were subjected to SDS-polyacrylamide gel electrophoresis. a. *sus*6(626); b. *sus*6(626) without ϕ 29 DNA-protein p3; c. *sus*6(626) + *sus*3(91); d. *sus*17(741); e. *sus*17(741) without ϕ 29 DNA-protein p3; f. *sus*17(741) + *sus*3(91); g. *sus*3(91); h. Markers of known molecular weight (the figures indicate K daltons).

the *B. subtilis* DNA polymerase III (35,36) had no effect on the formation of the initiation complex. A concentration of ddATP in 320-fold excess over the dATP, essentially did not inhibit the initiation reaction. Rifampicin and chloramphenicol, inhibitors of transcription and translation, respectively, had no effect on the formation of the initiation complex.

ATP requirement for the formation of the initiation complex

As already reported (20) and shown in Table 2, ATP is required for the formation of the p3-dAMP initiation complex, the maximal activity being obtained at a concentration of 1 mM (not

Table 1. Effect of inhibitors of replication, transcription and translation on the formation of the p3-dAMP initiation complex

<u>Inhibitor</u>	<u>Concentration</u>	<u>Activity, %</u>
None	-	100
Nalidixic acid	100 $\mu\text{g/ml}$	106
	200 $\mu\text{g/ml}$	117
Novobiocin	50 $\mu\text{g/ml}$	95
	100 $\mu\text{g/ml}$	117
Aphidicolin	40 $\mu\text{g/ml}$	87
	100 $\mu\text{g/ml}$	97
6-(p-hydroxyphenylazo) uracil	25 $\mu\text{g/ml}$	106
	50 $\mu\text{g/ml}$	104
ddATP	40 μM	82
	80 μM	80
Rifampicin	10 $\mu\text{g/ml}$	105
	20 $\mu\text{g/ml}$	91
Chloramphenicol	50 $\mu\text{g/ml}$	97
	200 $\mu\text{g/ml}$	95

S40-fraction prepared from Ø29 wild-type infected cells was incubated with $\{\alpha\text{-}^{32}\text{P}\}$ dATP and ATP in the absence or presence of the different inhibitors as described in Materials and Methods. The ^{32}P -labelled p3-dAMP band obtained by SDS-polyacrylamide gel electrophoresis was quantitated both by densitometry of the autoradiographs obtained by exposure under linear conditions and by counting the band sliced out of the gel. The two measurements were in good agreement. 100% corresponds to the incorporation of 0.5 fmol of ^{32}P -dAMP.

shown), with a stimulation of about 20-fold over the control without nucleotide. ATP can be replaced by GTP, CTP, UTP, dGTP, dCTP and dTTP in the initiation reaction; the activity with dCTP is even higher than with ATP. ADP was also active in the formation of the initiation complex, although less efficiently than the ribo- and deoxyribonucleoside triphosphates indicated above. AMP was essentially inactive. To try to determine whether or not ATP hydrolysis is required, the non-hydrolyzable analogs of ATP, AMP-PNP and AMP-PCP were tested in the *in vitro* initiation reaction. The activity with 1 mM AMP-PNP was about 19% of that ob-

Table 2. Effect of ATP and other nucleotides in the formation of the p3-dAMP initiation complex

<u>Addition</u>	<u>Concentration, mM</u>	<u>Activity, %</u>
ATP	1	100
	0,1	49
GTP	1	75
	0,1	149
CTP	1	64
	0,1	97
UTP	1	88
	0,1	110
dGTP	1	85
	0,1	132
dCTP	1	200
	0,1	213
dTTP	1	42
	0,1	120
ADP	1	42
	0,1	6
AMP	1	4
	0,1	3
AMP-PNP	1	19
	0,1	6
AMP-PCP	1	10
	0,1	6
None		4

S40-fraction prepared from Ø29 wild-type infected cells, passed through a Sephadex G50 column to remove endogenous nucleotides, was incubated with $\{\alpha\text{-}^{32}\text{P}\}$ dATP in the absence or presence of the different nucleotides as described in Materials and Methods. The amount of labelled p3-dAMP complex obtained by SDS-polyacrylamide gel electrophoresis was quantitated as described in Table 1. 100% corresponds to the incorporation of 0.75 fmol of ^{32}P -dAMP.

tained with 1 mM ATP. AMP-PCP at a concentration of 1 mM was much less active, being the formation of the initiation complex 10% of the amount obtained with 1 mM ATP, only slightly higher than the reaction which takes place in the absence of nucleotide.

DISCUSSION

Protein p3, present in extracts from ϕ 29-infected B. subtilis, reacts in vitro with dATP in the presence of ϕ 29 DNA-protein p3 as a template and ATP, forming a p3-dAMP covalent complex (20). Therefore, as shown in this paper, extracts from sus3-infected B. subtilis should not give rise in vitro to the formation of the p3-dAMP initiation complex.

To determine whether any other viral protein is needed for the initiation reaction, extracts from B. subtilis infected with sus or ts mutants in genes known to be required for ϕ 29 DNA replication were tested. When extracts from cells infected with two different sus mutants in gene 2 were used, no formation of the p3-dAMP complex occurred unless supplemented with a DNA-free fraction from sus3-infected cells and ϕ 29 DNA-protein p3 as template. Moreover, extracts prepared from cells infected at 44°C with a ts2 mutant were inactive in the formation of the initiation complex, but they could be complemented by addition of the sus3-fraction. As expected, extracts from cells infected at 30°C with the ts2 mutant were active in the formation of the initiation complex, in the absence or presence of the sus3-fraction. These results indicate that the gene 2 product is required for the initiation reaction, in agreement with the finding that in vivo gene 2 is involved in replication in an initiation step (12). The gene 2 product has been characterized as a protein of molecular weight 67 000 (37) and probably corresponds to the open reading frame 7 in the sequence of the left early region of ϕ 29 DNA (38), which would code for a protein of molecular weight 66 500. The gene 2 product was shown to be required in vivo for the attachment of phage ϕ 29 DNA to the host cell membrane (39, 40). In addition to this role, it is likely that the gene 2 product has some other role in the initiation reaction since membranes do not seem to be required in vitro for the formation of the initiation complex (20,21).

Extracts prepared from cells infected with sus mutants in genes 6 and 17 gave rise to the formation of the p3-dAMP initiation complex, suggesting that the corresponding gene products are not involved in the initiation reaction. Regarding the gene 17 product it has to be pointed out that the two mutants in gene

17 available, used in the experiments, are leaky (11; our unpublished results) and produce a small amount of synthesis of viral DNA. The possibility that some protein p17 had been synthesized or that a bacterial protein replaces the gene 17 product cannot be ruled out. In the case of the reaction with extracts from cells infected at 30°C or 44°C with mutant ts5(17) initiation complex was formed, although stimulation by addition of the sus3-fraction occurred. A similar stimulation by addition of the sus3-fraction was also observed with a DEAE-fraction prepared from wild-type-infected cells (unpublished results). The above results suggest that the gene 5 product is not required in the formation of the initiation complex, although it is possible that the mutation in protein p5, synthesized in the cells infected at 44°C with mutant ts5(17), is reversible and therefore, the protein could be active in the in vitro experiment carried out at 30°C. Unfortunately, there is essentially no formation of initiation complex in vitro at 42°C (unpublished results) and the above possibility cannot be excluded. Also, no sus mutation in gene 5 is presently available.

Inhibitors of DNA gyrase, DNA polymerase III or RNA polymerase were tested in vitro and shown not to affect the formation of the initiation complex. Aphidicolin, reported to inhibit Ø29 DNA replication in vivo (34) and ddATP, an inhibitor of DNA elongation, had little effect on the formation of the initiation complex. The last result suggests not only, as it has been shown (20), that the initiation and elongation are different events in Ø29 DNA replication, but that the initiation reaction is very selective for the 2'-deoxy,3'-hydroxy nucleotide, possibly because of the function of the initiation complex as a supplier of a free 3'-OH group for elongation.

ATP is needed in the initiation reaction, although it can be replaced by all ribo- and deoxyribonucleoside triphosphates tested. Similarly, in the formation of the initiation complex between the adenovirus preterminal protein and dCMP, ATP can be replaced by dATP, dGTP, GTP and UTP (41). ADP, but not AMP, was also active in the formation of the Ø29 initiation complex. The low stimulation obtained with the non-hydrolyzable analogs of ATP, AMP-PNP and AMP-PCP suggests that ATP hydrolysis might be

required in the initiation reaction; however, further studies with the purified proteins will be needed to answer this question. No ATPase activity has been detected in protein p3 purified (Prieto et al., in preparation) from extracts from E. coli transformed with the recombinant plasmid pKC30 A1 containing gene 3 (42), but other protein(s) involved in the initiation reaction, such as protein p2, might have that activity.

The precursor of the adenovirus terminal protein has been purified in a functional form and it was shown to copurify with a DNA polymerase activity different from DNA polymerases α , β or γ (43). More recently, Lichy et al. (44) could separate the adenovirus terminal protein precursor from the associated DNA polymerase activity and showed that both proteins are involved in the initiation reaction. In addition, a host factor has been purified and shown to stimulate the formation of the initiation complex in vitro, being an absolute requirement when the adenovirus DNA binding protein is present (45). DNA polymerase activity does not copurify with protein p3 (Prieto et al., in preparation). Whether or not the gene 2 product has DNA polymerase activity remains an open question. Cloning of gene 2 to overproduce and purify the protein is in progress.

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