Bacteriophage and host mutants causing the rolling-circle λ DNA replication early after infection

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Abstract There are two modes of bacteriophage λ DNA replication during its lytic development in *Escherichia coli* cells. The circle-to-circle (θ) replication predominates at early stages of the phage growth, whereas rolling-circle (σ) replication occurs late after infection to produce long concatemers that serve as substrates for packaging of λ DNA into phage proheads. The mechanism regulating the switch from θ to σ replication remains unknown. Our previous genetic studies indicated that the bacteriophage λ *P*ts*1*π*A66* mutant cannot replicate at 43°C in the wild-type *E. coli* host, but it can replicate in the *dnaA46*(ts) mutant. Density shift experiments suggested that the parental DNA molecules of the infecting phage enter σ replication. Here, using electron microscopy, we demonstrate that as soon as 5 min after infection of the *dnaA46*(ts) mutant by the λ*P*ts*1*π*A66* phage at 43°C, the σ replication intermediates are highly predominant over θ replication intermediates, contrary to the wild-type conditions (wild-type bacteria infected with the λP^+ phage). The initiation of replication of the λ*P*ts*1*π*A66* mutant at 43°C was strongly inhibited in the *dnaA*$^+$ host, as demonstrated by electron microscopy and by pulse-labeling of the phage-derived plasmid replicon. Implications for the mechanism of the regulation of the switch from θ to σ replication mode are discussed.

Key words: DNA replication (bacteriophage λ); Mutant π (bacteriophage λ); Early and late replication mode (bacteriophage λ); Electron microscopy; *dnaA* gene (*Escherichia coli*)

1. Introduction

Upon infection of its host, *Escherichia coli*, bacteriophage λ can follow one of the two alternative developmental pathways: lytic or lysogenic (for a review see [1]). During the lytic development, one of the crucial processes is phage DNA replication. Early after infection, the replication proceeds according to the circle-to-circle (θ) mechanism, whereas later it is switched to the rolling-circle (σ) mode (for a review see [2]). The σ replication produces long concatemers of λ DNA that are subsequently used for packaging of the phage genome units into proheads of the capsids. The regulation of the switch from θ to σ replication of λ DNA remains unknown. However, the control of this switch seems to be an important issue of general meaning since bacteriophage λ serves as a model in molecular biology studies, including regulation of DNA replication and viral development. In fact, results obtained with the bacteriophage λ model were successfully employed in studies on eukaryotic DNA replication (for a review see [3]), and life cycles (including DNA replication) of some eukaryotic viruses (e.g. herpes simplex virus) resemble that of bacteriophage λ [4].

To understand the molecular bases of the regulation of the switch from θ to σ replication of λ DNA, it was very important to find mutants altered in the control of this process. We have previously demonstrated that bacteriophage λ*P*ts*1*π*A66* mutant, unable to replicate in the wild-type host at elevated temperatures, efficiently produces progeny in an *E. coli* temperature-sensitive *dnaA46* mutant at 43°C [5,6]. Density shift experiments suggested that parental DNA molecules of the infecting phage may enter σ replication shortly after infection in these conditions [5]. These findings may be important steps in understanding the mechanism of regulation of the switch from θ to σ replication. However, suggestions mentioned above should be confirmed directly. Therefore, the aim of this work was to study the replication of the λ*P*ts*1*π*A66* phage DNA early after infection of the wild-type and *dnaA46*(ts) hosts at 43°C using electron microscopy, a technique that can demonstrate DNA replication intermediates in the most direct way.

2. Materials and methods

2.1. Bacterial strains, phages and plasmid

E. coli MG1655 (wild-type) [7], BM1 (*dnaA46*) [5,8] and QD5003 (*supF phx*) [9] strains were used. Bacteriophages λ*red3cI857S7* [5] and λ*red3cI857P*ts*1*π*A66S7* [5] were employed. The *P*ts*1*π*A66* allele bears two mutations: *P*ts*1* (a C to G substitution at nucleotide position 202 in the *P* gene, causing Arg to Gly substitution at amino acid position 68 in the P protein), responsible for the temperature-sensitive phenotype, and π*A66* (a G to T substitution at nucleotide position 410 in the *P* gene, causing Arg to Leu substitution at amino acid position 137 in the P protein), responsible for the π phenotype [10]. Plasmid pGW2 is a bacteriophage λ-derived plasmid bearing the *P*ts*1*π*A66* allele [11].

2.2. Investigation of kinetics of bacteriophage λ lytic development

Lytic development of bacteriophages was investigated in one-step growth experiments according to a previously described method [5]. Briefly, bacteria growing exponentially in LB medium at 30°C were centrifuged and resuspended in the fresh medium. Following incubation for 60 min at 43°C, NaN_3 was added to final concentration of 3 mM (in order to prevent unsynchronized phage development) and a phage lysate was added to multiplicity of infection (m.o.i.) of 5. Adsorption was carried out for 5 min at 43°C. After centrifugation, the bacterial pellet was resuspended in the same medium (with NaN_3) containing anti-λ serum and incubated for 5 min at 43°C to neutralize

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unadsorbed phages. The suspension was then diluted 1000-fold with the pre-warmed (to 43°C) medium (devoid of NaN_3) and aerated in a water-bath shaker. The number of infective centers was estimated on the basis of the number of cells in the mixture and the number of added phages. The number of intracellular progeny phages was estimated by plating the chloroform-treated samples of the infected culture withdrawn at different times, using QD5003 strain as a host. The ratio of the number of progeny phages to the number of infective centers was calculated for each sample.

2.3. Preparation of phage DNA samples for electron microscopy

Bacteria were grown at 30°C in the heavy medium (containing [^{13}C]glucose and [^{15}N]H_4Cl; for detailed description of this medium see [5]) in the presence of [^{14}C]thymidine (0.1 μCi/ml) to mid-log phase. Following centrifugation (2000×*g*, 10 min) of 10 ml of such a culture, the bacterial pellet was washed with the TM buffer (10 mM Tris–HCl pH 7.2, 10 mM $MgSO_4$) and resuspended in 1 ml of the same buffer. After 60 min incubation at 43°C, a lysate of the [^{3}H]thymidine-labeled phage λ*red3cI857S7* (3.4×10^{-5} cpm/pfu) or λ*red3cI857P*ts*1*π*A66S7* (2.7×10^{-5} cpm/pfu) was added to m.o.i. of 5, and the incubation was continued for 5 min. Then, 10 ml of the pre-warmed (to 43°C) LB medium was added (start of the phage development in the cells), and after another 5 min 0.5 ml of 1 mM NaN_3 was added and the culture was placed into an ice-bath. Following centrifugation (2000×*g*, 10 min, 4°C), the bacterial pellet was washed with 10 mM NaN_3 in 0.9% NaCl, and resuspended in 0.5 ml of TEN buffer (10 mM Tris–HCl pH 8.0, 20 mM EDTA, 10 mM NaN_3) containing 1 mg/ml lysozyme. After 15 min incubation at 37°C, 0.5 ml of 2% SDS were added, and incubation was continued for another 15 min at 65°C. Then, 20 μl of 20 mg/ml proteinase K solution were added and the sample was incubated for 60 min at 37°C. This mixture was added to the solution prepared by dissolving 6.4 g of CsCl in 4 ml of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). After ultracentrifugation (95 000×*g*, 70 h, 20°C) in the angle rotor (Beckman Ti50), five drop fractions were collected from the bottom of the tube. Radioactivity of 1/10 of the volume of each fraction was measured in a scintillation counter. Typical radioactivity profile is presented in Fig. 1. Fractions containing phage DNA ([^{3}H]thymidine peak) were used for electron microscopy studies.

2.4. Electron microscopy

Phage DNA samples obtained as described in Section 2.3 were dialyzed against TE buffer, and prepared for electron microscopy as described earlier [12]. Randomly selected molecules of the length equal to λ DNA or longer (in the case of replication intermediates) were analyzed from each sample.

2.5. Measurement of plasmid DNA synthesis

The experiments were performed as described previously [13]. Briefly, bacteria were grown in a minimal medium to mid-log phase. At indicated times the samples of equal cell mass were withdrawn, the volume was adjusted to 5 ml with the same medium, and pulse-labeling was performed with [^{3}H]thymidine. Following isolation of plasmid DNA and agarose gel electrophoresis, appropriate gel fragments containing plasmid bands were cut out, agarose was dissolved in 2 M HCl at 60°C, and radioactivity of the samples was measured in a scintillation counter.

3. Results

We investigated DNA replication of bacteriophage λ*red3-cI857P*ts*1*π*A66S7* (called λ*P*ts*1*π*A66* in further text) early after infection of the *E. coli dnaA*$^+$ and *dnaA46*(ts) hosts at 43°C. As a control of the wild-type conditions of a host infected by a phage, the wild-type *E. coli* strain and λ*red3-cI857S7* phage (called λP^+ in further text) were used. The *red3* mutation was used to eliminate the phage general recombination system as recombination intermediates could potentially complicate the analysis of λ DNA molecules in electron microscope. We found that the *red3* mutation still allows the effective lytic development of both phages in the *dnaA46*(ts) mutant at 43°C. The results of one-step growth experiments

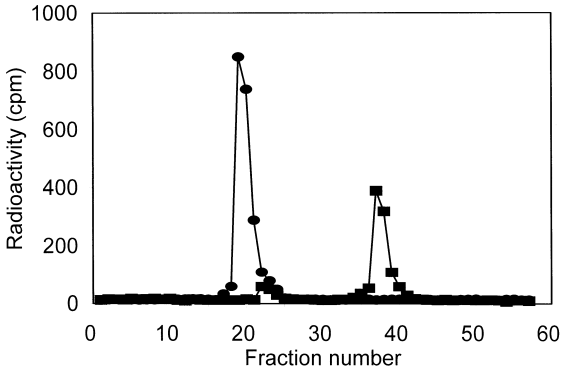

Fig. 1. Separation of [^{14}C]thymidine-labeled 'heavy' *E. coli* chromosomal DNA from [^{3}H]thymidine-labeled 'light' bacteriophage λ DNA after ultracentrifugation in the CsCl density gradient as described in Section 2.3. Radioactivity of ^{14}C (circles) and ^{3}H (squares) is presented.

with the λ*red3* mutants presented in Fig. 2 are comparable to those reported previously for analogous λ*red*$^+$ phages [5,6], though the values of the burst size of the λ*red3* mutants were about two times lower than those of the λ*red*$^+$ phages (data not shown). Moreover, in accordance to previously reported results [5,6], the *red3* derivative of the λP^+ phage, but not that of the λP*ts1*π*A66* phage, grew efficiently in the *dnaA*$^+$ host at 43°C (data not shown).

In order to determine the mode of phage DNA replication, following infection, phage DNA was separated from the host chromosome as described in Section 2.3 and depicted in Fig. 1, and samples were investigated using electron microscopy. Only circular λ DNA molecules and phage DNA replication intermediates were considered. We found that in the *dnaA46*(ts) host infected with phage λ*P*ts*1*π*A66* at 43°C, the rolling-circle replication intermediates are highly predominant over θ-like structures at the time after infection as short as 5 min (Table 1). The σ replication intermediates contained DNA tails of lengths from slightly more than one λ DNA unit to several λ genomes. This is in a strong contrast to the results of the infection of the wild-type cells by the λP^+ phage, where θ-like structures, but not σ-like structures, were abundant 5 min after infection (Table 1). Similar results with

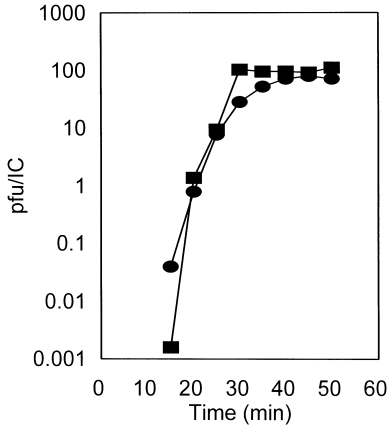

Fig. 2. Lytic development of phages λ*red3cI857S7* (circles) and λ*red3cI857P*ts*1*π*A66S7* (squares) in the *dnaA46*(ts) mutant at 43°C. The results are presented as plaque forming units per infective center (pfu/IC).

Table 1
Types of λ DNA molecules found in samples withdrawn from *E. coli* cultures infected with λ phages at 43°C, 5 min after infection

Type of λ DNA molecules	Analyzed molecules (%)	
	λ*red3cI857*Pts/π*A66S7* in *E. coli dnaA46*(ts) host	λ*red3cI857S7* in *E. coli dnaA*$^+$ host
Circular λ genomes	61	83
θ replication intermediates	4	15
σ replication intermediates	35	2

wild-type bacteria infected by wild-type λ phage were reported previously [14]. In those studies, rolling-circle replication intermediates started to predominate at significantly later times after infection [14]. We did not find any replication intermediates among 100 λ DNA molecules randomly selected in the samples taken from the *dnaA*$^+$ host infected with the λ*P*ts*1*π*A66* phage at 43°C (data not shown).

The lack of replication intermediates among the 100 DNA molecules observed in the case of λ*P*ts*1*π*A66* infection of the *dnaA*$^+$ host at 43°C may explain previous observations that this phage mutant cannot form progeny in these conditions. However, to test whether replication starting from *ori*λ is inhibited in wild-type host in the presence of the *P*ts*1*π*A66* allele, we investigated kinetics of replication of the λ*P*ts*1*π*A66* phage-derived plasmid, pGW2. This plasmid contains a replication region of the phage λ*P*ts*1*π*A66* (i.e. the p_R promoter, and *cro*$^+$, *cII*$^+$, *O*$^+$, *P*ts*1*π*A66* and *ren*$^+$ alleles) and a kanamycin-resistance gene. Wild-type *E. coli* cells bearing plasmid pGW2 were grown at 30°C and then the culture was shifted to 43°C. Samples of the same cell mass were withdrawn at indicated times and after pulse-labeling with [^{3}H]thymidine, plasmid DNA was isolated and radioactivity incorporated into this DNA was measured. Therefore, the constant value of the measured radioactivity indicated plasmid DNA replication, and inhibition of plasmid DNA replication should result in a decrease in the amount of incorporated radioactivity. We found that shortly after the temperature shift, the efficiency of DNA synthesis of plasmid pGW2 is dramatically decreased (Fig. 3A). These results indicate that replication from *ori*λ is strongly inhibited in these conditions indeed.

In analogous experiments performed with the *dnaA46*(ts) host bearing plasmid pGW2 we also observed a reduction in the [^{3}H]thymidine incorporation into plasmid DNA after a temperature shift, though somewhat less efficient than in the case of the *dnaA*$^+$ host (Fig. 3B). This reduction may be explained on the basis of previous findings that dysfunction of *dnaA* results in inhibition of replication of any λ plasmids, even those that can replicate in certain *dnaA*(ts) mutants at permissive temperatures, contrary to the situation observed in λ phages [5,6,10,15]. One explanation of this phenomenon might be that if the rolling-circle replication of λ*P*ts*1*π*A66* plasmid predominates in the *dnaA46*(ts) host at 43°C, like in the case of the phage λ*P*ts*1*π*A66* DNA (see Table 1), the σ-like plasmid structures could be efficiently degraded in the host cells due to a lack of the *gam* gene that encodes a inhibitor of the RecBC nuclease which prevents degradation of phage λ DNA rolling-circle replication intermediates (for a review see [2]).

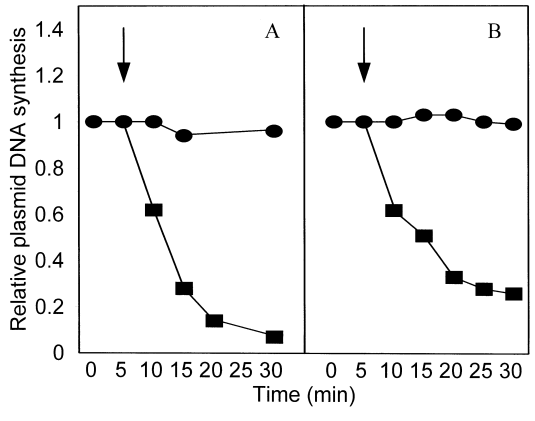

Fig. 3. Replication of the λ*P*ts*1*π*A66* plasmid (pGW2) in the *dnaA*$^+$ (A) and *dnaA46*(ts) (B) hosts at 30°C (circles), and after a shift to 43°C (squares) as measured by pulse-labeling with [^{3}H]thymidine. Times of the temperature shifts are indicated by arrows.

4. Discussion

The first step in bacteriophage λ DNA replication is binding of the phage-encoded O protein to the *ori*λ region and formation of a so called O-some. Then, the P-DnaB protein complex binds to the O-some, and the nucleoprotein preprimosome structure is formed. Since the P protein binds DnaB helicase strongly and inhibits its enzymatic activities, the action of the molecular chaperones DnaK, DnaJ and GrpE is necessary for liberation of the DnaB helicase from P-mediated inhibition. At the same time, the complex is properly positioned at the origin. This process depends on the transcriptional activation of *ori*λ, a transcription starting from the p_R promoter and proceeding at or near the origin region. The p_R promoter is stimulated by the host-encoded DnaA protein, thus DnaA may indirectly regulate initiation of replication from *ori*λ (for reviews see [1,2]).

The π mutants have been isolated many years ago as phage suppressors of mutations in *E. coli dnaB*, *dnaK*, *dnaJ* and *grpE* genes [16]. Mutations of the π type have been mapped in the λ *P* gene [16,17], and more recent biochemical studies demonstrated that a product of one of the π allele (*P*ts*1*π*A66*) reveals weaker affinity to DnaB than the wild-type P protein [18].

Our previous studies demonstrated that bacteriophage λ*P*ts*1*π*A66* is unable to replicate in wild-type *E. coli* cells at 43°C, but this phage produces progeny at this temperature in the *dnaA46*(ts) host [5,6]. Here we demonstrate that replication of λ*P*ts*1*π*A66* phage DNA in the mutant host proceeds predominantly according to the rolling-circle mode even at very early stages of infection. This finding may provide some important information for understanding of the regulation of the switch from θ to σ replication mode. According to the predominant hypothesis, the bacteriophage λ rolling-circle replication may be preceded by one round of the unidirectional θ replication initiated at *ori*λ, followed by displacement of the 5′ end of the newly synthesized strand by its growing 3′ end [19]. However, it is not known what triggers the change from bidirectional θ replication (occurring early after infec-

tion) to the unidirectional θ replication followed, after one round, by the rolling-circle mechanism. The appearance of abundant rolling-circle replication intermediates early after phage λ*P*ts*1*π*A66* infection of the *dnaA46*(ts) host at 43°C suggests that in this case the unidirectional θ replication starts immediately after the infection, and after one round is followed by the σ replication. One possible explanation of this phenomenon is that putative premature partial disassembly of the very weak λ replication complex allows for establishment of only one replication fork, and thus the replication proceeds unidirectionally. On the other hand, when DnaA-mediated stimulation of the p_R promoter is impaired in the *dnaA*46(ts) mutant at 43°C, the less efficient transcriptional activation of *ori*λ, that is coupled with the rearrangement of the preprimosomal structure by DnaK, DnaJ and GrpE proteins [15], may be sufficient for proper installation of only one replication complex at the origin.

The *P*ts1π*A66* allele bears two mutations (*P*ts*1*, responsible for a temperature-sensitive phenotype, and π*A66*, responsible for the π phenotype) [10]. It was therefore interesting to ask which mutation is responsible for the specific phenotype of the λ*P*ts*1*π*A66* phage, investigated in this work. The phage harboring a single π*A66* mutation in the *P* gene was previously found to be able to yield progeny in a wild-type host at 43°C [10]. However, λ plasmids bearing the π*A66* mutation can replicate in the *E. coli dnaA46*(ts) hosts at permissive temperature (30°), but not at 43°C, contrary to plasmids devoid of a mutation of the π type which are unable to transform these hosts even at 30°C [10,15]. On the other hand, the inhibition of phage lytic development in *dnaA*$^+$, but not in *dnaA46*(ts), cells growing at 43°C was reported exclusively for λ*P*ts*1*π*A66* [5,6]. Therefore, it seems that the very characteristic phage phenotype, described in this report, results from specific properties of the changed P protein defined by both mutations present in the *P*ts*1*π*A66* allele.

In conclusion, it seems that transcriptional activation of *ori*λ and stability of the preprimosomal complex are crucial processes in the regulation of the switch from an early to a late replication of bacteriophage λ DNA. Both processes have been demonstrated to be affected by the host *dnaA* gene product [20,21]. Thus, DnaA may be considered a main regulator of λ DNA replication mode. The use of the λ*P*ts*1*π*A66* mutant allowed to identify the processes crucial in the regulation of the switch from θ to σ replication. Further studies should lead to understanding how this switch is controlled in the wild-type phage at a particular time after infection.

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